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Functional Analysis of the Mitochondrial Outer Membrane Protein TOM70 in *Neurospora crassa*

by

Leslie Ian Grad

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

in

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Leslie Grad

205-6119 Cooney Road
Richmond, B.C.
Canada
V6Y 4B7

Sept. 28/98

University of Alberta

Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled Functional Analysis of the Mitochondrial Outer Membrane Protein TOM70 in *Neurospora crassa* submitted by Leslie Ian Grad in partial fulfillment of the requirements for the degree of Master of Science in Molecular Biology and Genetics.

Frank Nargang

Supervisor: Dr. Frank Nargang

John B Bell

Dr. John Bell

Bernard Lemire

Dr. Bernard Lemire

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for my family and friends

Abstract

Over 95% of the proteins in mitochondria are the products of nuclear genes and are synthesized in the cytosol as mitochondrial preproteins. Mitochondrial biogenesis requires that preproteins be efficiently imported into existing organelles. The TOM (translocase of the mitochondrial outer membrane) complex achieves recognition, unfolding, insertion, and translocation of preproteins at the mitochondrial outer membrane. The largest component of this complex, TOM70, is an integral outer membrane protein with a large cytosolic domain thought to serve as a receptor for a specific group of preproteins. To investigate the functional role of TOM70 in *Neurospora crassa*, the *tom70* gene was inactivated using the natural phenomenon of repeat induced point mutation (RIP). In contrast to the lethal phenotypes of null mutations in the *tom20* or *tom22* genes, which encode two other receptor components of the TOM complex, the effects of *tom70* inactivation are relatively mild. *tom70*^{RIP} strains are viable, but have a mild growth defect and conidiate poorly. *tom70*^{RIP} strains contain enlarged mitochondria with fragile outer membranes that are susceptible to damage during the standard isolation procedure. Assays of preprotein import into isolated mitochondria demonstrate a defect in the import efficiency of specific preproteins, such as the ATP/ADP carrier, into TOM70-deficient mitochondria. However, import of most other preproteins does not differ significantly from wild-type. These data support the view that TOM70 plays a specific role in mitochondrial preprotein import. The protein is also required for the maintenance of proper mitochondrial morphology and mitochondrial outer membrane integrity, though it has not been determined if these defects are directly or indirectly due to lack of TOM70.

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List of Abbreviations

α-MPP	α-subunit of the matrix processing peptidase
A	adenine
AAC	ATP/ADP carrier
ADP	adenosine diphosphate
ala	alanine
ampR	gene conferring ampicillin-resistance
ATP	adenosine triphosphate
ATPase	adenosine triphosphotase
BSA	bovine serum albumin
bp	base pair
C	cytosine
CCHL	cytochrome *c* heme lyase
cDNA	complimentary deoxyribonucleic acid
$\Delta\Psi$	membrane potential
DIG	digoxigenin
DNA	deoxyribonucleic acid
DnaJ	deoxyribonucleic acid protein J
DnaK	deoxyribonucleic acid protein K
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
$F_1\beta$	β-subunit of the F_1 ATPase
G	guanine
g	gram
glu	glutamic acid
gly	glycine
h	hour
his_6	hexahistidinyl
hph	gene encoding bacterial hygromycin phosphotransferase
Hsp10	heat shock protein of 10 kDa
Hsp60	heat shock protein of 60 kDa
Hsp70	heat shock protein of 70 kDa
hygR	gene conferring hygromycin-resistance
IM	inner membrane
IMS	intermembrane space
Isp6	import site protein of 6 kDa
Isp42	import site protein of 42 kDa
Isp45	import site protein of 45 kDa
kb	kilobase
kDa	kilodaltons
lys	lysine
µg	microgram
µl	microlitre
M	molar
Mas6p	mitochondrial assembly protein #6 of 23 kDa

Mas20p	mitochondrial assembly protein of 20 kDa
Mas22p	mitochondrial assembly protein of 22 kDa
Mas70p	mitochondrial assembly protein of 70 kDa
MDJ1	mitochondrial DnaJ protein #1
Mdm1p	mitochondrial distribution and morphology protein #1
Mdm10p	mitochondrial distribution and morphology protein #12
mg	milligram
MGE	mitochondrial GrpE
mGrpE	mitochondrial GrpE
MIM17	mitochondrial inner membrane protein of 17 kDa
MIM23	mitochondrial inner membrane protein of 23 kDa
min	minute
ml	millilitre
mM	millimolar
Mmm1p	maintenance of mitochondrial membrane protein #1
MOM8a	mitochondrial outer membrane protein a of 8 kDa
Mom8b	mitochondrial outer membrane protein b of 8 kDa
MOM19	mitochondrial outer membrane protein of 19 kDa
MOM22	mitochondrial outer membrane protein of 22 kDa
MOM38	mitochondrial outer membrane protein of 38 kDa
MOM72	mitochondrial outer membrane protein of 72 kDa
MOPS	3-(N-morpholino) propanesulfonic acid
Mpi1p	mitochondrial protein import protein #1
MPP	matrix processing peptidase
MTF1	mitochondrial transcription factor protein #1
mRNA	messenger ribonulceic acid
Mrs5	mitochondrial RNA splicing protein #5
Mrs11	mitochondrial RNA splicing protein #11
MSF	mitochondrial import stimulating factor
Mpi1p	mitochondrial protein import protein #1
mtHsp70	mitochondrial Hsp70
NADH	nicotinamide adenine dinucleotide, reduced form
N. crassa	*Neurospora crassa*
NEM	N-ethylmaleimide
nic	nicotinamide
OM	outer membrane
PAGE	polyacrylamide gel electrophoresis
pan	D-pantothenic acid
P. anserina	*Podospora anserina*
PBF	presequence binding factor
PCR	polymerase chain reaction
PK	proteinase K
PMSF	phenylmethylsulfonyl fluoride
RIP	repeat induced point mutation
rpm	revolutions per minute
Sms1	serine-rich multi-copy repressor protein #1

Ssc1	stress seventy subfamily C protein #1
SDS	sodium dodecyl sulfate
SEM	sucrose, EDTA, MOPS
SEMP	sucrose, EDTA, MOPS, PMSF
T	thymine
TAE	tris, acetate, EDTA
TIM10	translocase of the mitochondrial inner membrane protein of 10 kDa
TIM12	translocase of the mitochondrial inner membrane protein of 12 kDa
TIM17	translocase of the mitochondrial inner membrane protein of 17 kDa
TIM22	translocase of the mitochondrial inner membrane protein of 22 kDa
TIM23	translocase of the mitochondrial inner membrane protein of 23 kDa
TIM44	translocase of the mitochondrial inner membrane protein of 44 kDa
TOM5	translocase of the mitochondrial outer membrane protein of 5 kDa
tom6	gene encoding TOM6
TOM6	translocase of the mitochondrial outer membrane protein of 6 kDa
tom7	gene encoding TOM7
TOM7	translocase of the mitochondrial outer membrane protein of 7 kDa
TOM20	translocase of the mitochondrial outer membrane protein of 20 kDa
TOM22	translocase of the mitochondrial outer membrane protein of 22 kDa
TOM37	translocase of the mitochondrial outer membrane protein of 37 kDa
TOM40	translocase of the mitochondrial outer membrane protein of 40 kDa
tom70	gene encoding TOM70
TOM70	translocase of the mitochondrial outer membrane protein of 70 kDa
tom70^{RIP}	RIPed allele of *tom70*
tom71	gene encoding TOM71
TOM71	translocase of the mitochondrial outer membrane protein of 71 kDa
Tom72	translocase of the mitochondrial outer membrane protein of 72 kDa
Tris	tris (hydroxymethyl) aminomethane
trp	gene encoding tryptophan biosynthetic protein
YDJ1	gene encoding yeast DnaJ protein #1

Ydj1p	yeast DnaJ protein #1
Yge1p	yeast GrpE protein 1
yme1	yeast mitochondrial DNA escape gene #1

Chapter 1: Preprotein import into mitochondria

1·1 General introduction

Mitochondria are present in almost all eukaryotes and are essential organelles in these organisms. Their primary function is to provide energy for the cell via oxidative phosphorylation, but they harbour a variety of metabolic pathways as well (reviewed in Tzagoloff, 1982). The organelles consist of an outer membrane, an inner membrane, an intermembrane space, and an internal compartment called the matrix. Mitochondria possess their own genome and protein synthesis machinery, but only a small fraction of mitochondrial proteins, mostly constituents of the inner membrane, are actually synthesized in the matrix (Attardi and Schatz, 1988; Collins, 1990). The remaining ~95% of mitochondrial proteins are encoded in the nucleus and are synthesized on cytosolic ribosomes as mitochondrial preproteins, or precursors. Nuclear-encoded proteins destined for the mitochondria contain a mitochondrial targeting signal. Most of these occur as an N-terminal presequence, but some occur as internal targeting signals. Such sequences are necessary for directing proteins to the mitochondrial surface and for sorting them to the proper mitochondrial subcompartment (Hurt and van Loon, 1986; Schatz, 1987). Mitochondria arise by growth and division of pre-existing mitochondria. New mitochondrial protein constituents that are encoded in the nucleus must be imported from the cytosol into growing mitochondria. Therefore, the targeting, uptake, and proper sorting of mitochondrial proteins are essential aspects of mitochondrial biogenesis.

Investigations in the last decade have identified many components involved in mitochondrial protein import (for reviews see Neupert, 1997; Pfanner *et al.*, 1997). Molecular chaperones in the cytosol facilitate the

targeting of preproteins to the mitochondrial surface and also maintain them in a conformation favourable for translocation. The TOM complex (translocase of the mitochondrial outer membrane) contains surface receptors for the recognition of mitochondrial preproteins and a translocation pore through which the preproteins traverse the outer membrane (Fig. 1-1). In the inner membrane, two separate TIM complexes (translocase of the mitochondrial Inner membrane) facilitate either the entry of preproteins to the matrix or their insertion into the inner membrane in the presence of a membrane potential (Fig. 1-1). Finally, matrix chaperones are responsible for driving translocation through the inner membrane and folding proteins to their native conformation.

The protein components involved in mitochondrial protein import have been investigated primarily in *Neurospora crassa* and *Saccharomyces cerevisiae* (yeast), although homologues of some of these components have been identified in humans (Goping *et al.*, 1995; McBride *et al.*, 1996), rats (Komiya and Mihara, 1996), other filamentous fungi (Jamet-Vierny *et al.*, 1997), and plants (Jänsch *et al.*, 1998). The work in this thesis will focus on a component of the TOM complex in *N. crassa*, the mitochondrial outer membrane protein TOM70. The gene encoding TOM70 was inactivated and the resulting mutants were analyzed in order to determine the functional role of the protein in mitochondrial preprotein import.

1·2 Receptor-mediated mitochondrial protein import

1·2·1 Mitochondrial targeting signals

For most proteins, mitochondrial targeting signals are contained within N-terminal extensions that are removed upon import into the mitochondrion. Cleavable N-terminal presequences direct preproteins to the matrix space,

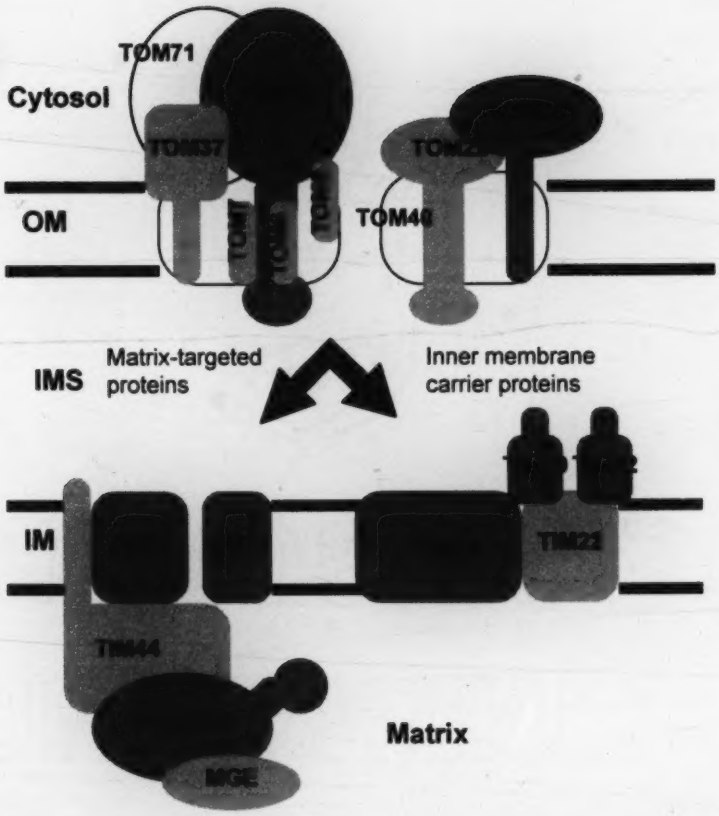

Figure 1-1. Schematic representation of components of the TOM and TIM complexes. Adapted from Neupert (1997) and Sirrenberg *et al.* (1998). All components present in this diagram have been identified in yeast. In *N. crassa*, TOM37 and TOM71 have not been identified and there is inconclusive evidence regarding the existence of TOM5 (Künkele *et al.*, 1998). Of the TIM components, only TIM17 has been identified in *N. crassa* (Tan and Nargang, unpublished). OM, outer membrane; IMS, intermembrane space; IM, inner membrane.

and are therefore also referred to as matrix-targeting sequences. Typical matrix-targeting presequences are about 20-60 amino acid residues in length with an abundance of positively charged amino acid residues, and a relatively high frequency of hydroxylated residues. These presequences are predicted to form amphipathic α-helices which are thought to play an important role in specific recognition by the mitochondrial import machinery and their subsequent translocation through the membranes (Lemire *et al.*, 1989; Roise and Schatz, 1988). It is thought that the receptor components of the TOM complex provide binding sites for both the hydrophobic and positively charged faces of the α-helical targeting sequence and directs the preprotein through the translocation pore. Alternatively, studies involving synthetic peptides corresponding to presequences have confirmed their predicted amphipathic structure in a membrane environment, and have shown interactions between presequences and phospholipid bilayers. This indicates that initial interaction of the presequence could occur by direct association with the mitochondrial outer membrane (Swanson and Roise, 1992; Zardeneta and Horowitz, 1992). Upon arrival into the matrix, presequences are removed by the matrix processing peptidase. A number of investigations involving chimeric protein fusions have shown that N-terminal presequences are necessary and sufficient to direct non-mitochondrial proteins into the matrix (Hartl and Neupert, 1990).

In contrast to matrix-targeting presequences, internal targeting signals are poorly characterized. Preproteins containing internal targeting information are not cleaved once imported into the mitochondrion. Internal signals are found in a majority of proteins that reside in the mitochondrial inner and outer membranes, including most of the components of the TIM and TOM complexes. An exception to this is the matrix-located yeast transcription

factor MTF1 which lacks a recognizable matrix-targeting sequence and presumably uses internal targeting information for its import into the matrix (Sanyal and Getz, 1995). The internal targeting information of inner membrane proteins such as the large family of carrier proteins, as well as certain components of the TIM complexes, such as TIM22, TIM17, and TIM23 have been investigated (Sirrenberg *et al.*, 1998; Bauer *et al.*, 1996; Sirrenberg *et al.*, 1996). The ATP/ADP carrier (AAC), the most abundant mitochondrial protein, does not contain a cleavable N-terminal presequence, but earlier investigations have shown that this and other members of the carrier family of proteins have more than one internal targeting signal (Liu *et al.*, 1988; Pfanner and Neupert, 1987). In addition to this, three repeat elements have been identified in carrier proteins, each of which contains a matrix-exposed sequence motif termed a 'carrier signature' (Palmieri, 1994; Saraste and Walker, 1982). A similar sequence has also been identified in yeast TIM22 (Sirrenberg *et al.*, 1996). These sequences appear to be involved in the insertion of these proteins into the mitochondrial inner membrane. The addition of a matrix targeting signal to the N-terminus of proteins with internal targeting information, such as mitochondrial outer membrane porin or the inner membrane uncoupling protein, overrides the normal targeting and sorting signals and the fusion proteins are imported into the matrix (Liu *et al.*, 1990; Sakaguchi *et al.*, 1992).

Targeting signals of outer membrane proteins have also been analyzed. In proteins anchored to the mitochondrial outer membrane by hydrophobic segments in the N-terminus, such as TOM70, a combination of the hydrophobicity of the transmembrane anchor and the nature of the flanking sequences determines targeting and membrane insertion (Steenaart *et al.*, 1996). Similarly, those outer membrane proteins anchored by

hydrophobic segments in the C-terminus appear to have their targeting information in the C-terminal region (Nguyen *et al.*, 1993). The precise mechanism of recognition and insertion of these mitochondrial proteins by the outer membrane import machinery is not well understood. TOM22, a receptor protein that spans the outer membrane via a central trans-membrane domain, utilizes a short positively charged segment just prior to the membrane-spanning domain for targeting. Removal of portions, or a reduction in the net negative charge of this segment reduces the efficiency of import of TOM22, but still allows it to assemble in the mitochondrial outer membrane (Rodriguez-Cousino *et al.*, 1998). Thus, TOM22 contains physically separate import and membrane anchor sequences.

1·2·2 Cytosolic factors

Nuclear-encoded preproteins can interact with cytosolic components once released from cytosolic ribosomes. During the time in transit between the ribosome from which it emerged and the mitochondrial surface, events may occur which affect the import competency of a preprotein (Cyr *et al.*, 1994). Aggregation or premature folding would preclude translocation through the pore (Eilers and Schatz, 1986). Premature degradation by cellular proteases possibly due to misfolding of the protein (Jentsch, 1992) would also decrease overall import of a preprotein. Cytosolic chaperones safeguard against these events and effectively extend the lifetime of the preprotein within the cytosol. A number of cytosolic chaperones have been identified that either preserve import competence or stimulate import. Cytosolic Hsp70, the eukaryotic homologue of bacterial DnaK, was identified as an important factor in mitochondrial import as well as import into other organelles. In yeast strains where the genes encoding isoforms of Hsp70

were deleted, mitochondrial protein import was impaired and preproteins would accumulate in the cytosol of mutant cells (Deshaies *et al.*, 1988). Hsp70 is thought to stabilize the denatured structure of preproteins in transit to the mitochondria (Gething and Sambrook, 1992), but does not interact specifically with receptors on the mitochondrial surface (Ellis, 1987). Another well characterized cytosolic chaperone is the Ydj1 protein, a farnesylated yeast homologue of bacterial DnaJ (Caplan and Douglas, 1991). Conditional mutants of the *YDJI* gene demonstrated growth defects and an impairment of mitochondrial import (Caplan *et al.*, 1992a; Caplan *et al.*, 1992b). It is thought that Hsp70 and Ydj1p interact with each other to maintain preproteins in an import competent state and deliver them to the mitochondrion. Another cytosolic factor, presequence binding factor (PBF), has been shown to bind specifically to cleavable presequences of mitochondrial preproteins (Murakami *et al.*, 1988). PBF is a protein factor with a molecular weight of 50 kDa purified from reticulocyte lysate. The ability of PBF to stimulate import was not dependent upon ATP hydrolysis and was resistant to treatment with N-ethylmaleimide (NEM). Import was stimulated further upon addition of Hsp70 suggesting PBF maintains the import competence of mitochondrial preproteins in conjunction with Hsp70 (Murakami and Mori, 1990).

One of the best characterized cytosolic factors involved in mitochondrial protein import is mitochondrial import stimulating factor (MSF; Mihara and Omura, 1996). MSF, a member of the 14-3-3 family of proteins (Alam *et al.*, 1994), is a heterodimer consisting of 30- and 32-kDa subunits purified from rat liver cytosol (Hachiya *et al.*, 1994). Initial studies have shown that MSF stimulates the import of preadrenodoxin precursor, synthesized from wheat germ lysate, into isolated mitochondria in an ATP-dependent and NEM-sensitive manner. MSF also stimulates the

mitochondrial import of a number of other preproteins, including those with cleavable presequences, such as presuperoxide dismutase and a yeast CoxIV-presequence-porin fusion, and also preproteins without presequences such as porin (Mihara and Omura, 1996). MSF can also recognize the targeting signal of preadrenodoxin as well as synthetic peptides that correspond to other mitochondrial targeting signals. The import stimulation activity of MSF was strongly impaired with NEM treatment, but its preprotein unfolding activity was unaffected. Unlike other cytosolic factors, MSF was shown not only to stimulate import, but also to interact with the surface receptors of the mitochondrial outer membrane (Hachiya *et al.*, 1995). A MSF-preprotein complex was shown to interact initially with the yeast mitochondrial outer membrane receptors TOM70 and TOM37 before releasing the preprotein in an ATP-dependent manner. The preprotein then proceeds to other receptors of the TOM complex before being inserted through the translocation pore. It has been suggested that import of preproteins can occur via two separate pathways, an ATP-dependent pathway utilizing MSF, and an ATP-independent pathway involving Hsp70 (Komiya *et al.*, 1996). The pathway used is dependent upon the nature of the preprotein. So far MSF has yet to be identified in either *N. crassa* or yeast, the two primary model systems used to investigate mitochondrial protein import. In addition, only one precursor, preadrenodoxin, has been demonstrated to form a complex with MSF *in vitro* (Hachiya *et al.*, 1994).

1·2·3 Translocase of the mitochondrial outer membrane: the TOM complex

1·2·3·1 Components of the TOM complex

The translocation machinery of the mitochondrial outer membrane in *N. crassa* and yeast is composed of at least six and nine different protein

subunits, respectively. A list of the known components of the TOM complex is presented in Table 1-1. Components are named based on a uniform nomenclature that reflects the consensus molecular weight of the protein (Pfanner *et al.*, 1996). Three proteins found in *N. crassa* and yeast, TOM20, TOM22, and TOM70 have large cytosolic domains, which are thought to interact with preproteins prior to insertion through the outer membrane. TOM20 has a small N-terminal transmembrane anchor followed by a cytosolic domain of ~17kDa. Homologues of TOM20 have also been found in humans (Goping *et al.*, 1995) and plants (Jänsch *et al.*, 1998) demonstrating that the protein is conserved throughout evolution. TOM22 is an integral protein that spans the outer membrane. In *N. crassa*, the N-terminal domain of the protein extends 85 amino acid residues into the cytosol has a single transmembrane domain of 20 amino acid residues, and a C-terminal intermembrane space domain of 45 residues. The cytosolic domain of TOM22 contains an abundance of negative charges (Kiebler *et al.*, 1993; Lithgow *et al.*, 1994a). A fourth putative receptor protein, TOM71, has been found only in yeast and is very closely related to TOM70 with 53% sequence identitiy and 70% similarity (Bömer *et al.*, 1996a; Schlossmann *et al.*, 1996). Both yeast TOM70 and TOM 71 have an ~65 kDa cytosolic domain and are anchored to the outer membrane by a small N-terminal transmembrane domain of approximately 20 amino acids (Millar and Shore, 1993). TOM70 and TOM71 both contain seven tetratricopeptide repeat (TPR) motifs while TOM20 appears to carry at least a single partial TPR motif (Haucke *et al.*, 1996; Moczko *et al.*, 1994). TPR motifs are found in a diverse family of proteins that include proteins involved in cell cycle regulation and RNA synthesis. The motifs are thought to mediate protein-protein interactions (Boguski *et al.*, 1990) and may play a role in interactions between TOM

Table 1-1. Components of the translocase of the mitochondrial outer membrane.

Component	Other names	Identified in yeast	Identified in *N.crassa*	Function
TOM20	MOM19 Mas20p	Yes	Yes	Primary preprotein surface receptor with TOM22 (Harkness *et al.*, 1994; Ramage *et al.*, 1993; Söllner *et al.*, 1990).
TOM22	MOM22 Mas22p	Yes	Yes	Primary preprotein receptor with TOM20; is essential in yeast and *N. crassa* (Kiebler *et al.*, 1990; Lithgow *et al.*, 1994a; Nargang *et al.*, 1995).
TOM40	MOM38 ISP42	Yes	Yes	Membrane embedded protein thought to form the translocation pore; is essential in yeast and *N. crassa* (Kiebler *et al.*, 1990; Vestweber and Schatz, 1989) Taylor *et al.*, unpublished).
TOM70	MOM72 Mas70p	Yes	Yes	Specialized surface receptor for carrier proteins (Hines *et al.*, 1990; Söllner *et al.*, 1990; Steger *et al.*, 1990).
TOM71	TOM72	Yes	No	Surface receptor with a similar function to that of TOM70 (Bömer *et al.*, 1996a; Schlossmann *et al.*, 1996).

TOM37	Mas37p	Yes	No	Surface receptor that acts in conjunction with TOM70 in yeast (Gratzer *et al.*, 1995).
TOM5	MOM8a	Yes	Yes*	May contribute to the structure of the translocation pore and provide a link between the surface receptors and the pore (Dietmeier *et al.*, 1997; Söllner *et al.*, 1992).
TOM6	Isp6p Mom8b	Yes	Yes	May contribute to the structure of the translocation channel; stabilizes interactions between the surface receptors and the pore (Cao and Douglas, 1995; Kassenbrock *et al.*, 1993).
TOM7	MOM7	Yes	Yes	May contribute to the structure of the translocation channel and modulates the dynamics of the surface receptors (Hönlinger *et al.*, 1996).

* Uncertain data for its existence in *N. crassa* as explained in Künkele *et al.* (1998).

proteins (Haucke *et al.*, 1996). A homologue of TOM70 has also been identified in the filamentous fungus *Podospora anserina* (Jamet-Vierny *et al.*, 1997). Another putative preprotein receptor, TOM37, which has only been identified in yeast, has a cytosolic domain and two predicted transmembrane segments (Gratzer *et al.*, 1995).

Along with the TOM components exposed to the cytosol, there are those that are more deeply embedded in the mitochondrial outer membrane. TOM40, a protein identified in both *N. crassa* and yeast is largely resistant to cleavage by extra-mitochondrial proteases and may exist as a β-barrel structure similar to those found in mitochondrial porins (Court *et al.*, 1995). Other membrane-embedded components of the TOM complex include TOM5, TOM6, and TOM7 which have been shown to co-immunoprecipitate with TOM20 or TOM40 (Hönlinger *et al.*, 1996; Moczko *et al.*, 1992), and have been found in highly purified TOM complex preparations (Künkele *et al.*, 1998). TOM5 possesses a single-membrane anchor at the C-terminus, and its N-terminal segment, which contains a net negative charge, is exposed to the cytosol. TOM5 and TOM6 have been shown to closely associate with TOM40 (Dietmeier *et al.*, 1997; Kassenbrock *et al.*, 1993).

The components of the TOM complex have been found to be in close proximity to each other. TOM20 and TOM70 can be cross-linked and co-immunoprecipitated in yeast (Haucke *et al.*, 1996). In *N. crassa* TOM22 can be cross-linked to TOM70 (Schlossmann and Neupert, 1995) and TOM20 (Mayer *et al.*, 1995b). TOM71 can be co-immunoprecipitated with TOM70, and to a lesser extent TOM40 (Schlossmann *et al.*, 1996). TOM70 has been shown to form a complex with TOM37 with a stoichiometry of 1:1 (Gratzer *et al.*, 1995). Only a fraction of total TOM70 in *N. crassa* is found in complex with the other components of the TOM complex, indicating the complex is

dynamic (Dekker *et al.*, 1996; Künkele *et al.*, 1998; Söllner *et al.*, 1992). This may suggest that TOM70 has a function other than that of a preprotein receptor.

Recently, the TOM complex of *N. crassa* has been purified and its physical structure has been determined by electron microscopy and imaging analysis (Künkele *et al.*, 1998). The TOM complex forms a cylindrical oligomer with a diameter of about 138 Å. Purified TOM complex particles contain two or three centres of stain-filled openings that are interpreted to be pores with an apparent diameter of 20 Å. The stoichiometry of TOM70, TOM40, TOM22, and TOM20, the main constituents of the outer membrane translocase, was determined to be roughly 1.5 : 8 : 3 : 2. TOM6, TOM7, and possibly TOM5, have also been identified in this purified complex (Künkele *et al.*, 1998; Dembowski *et al.*, manuscript in preparation), but their molar ratios could not be determined. These findings suggest that TOM40 and TOM22, the only essential subunits of the mitochondrial outer membrane preprotein import machinery in *N. crassa* and yeast, are the major constituents of the translocation pore. In addition, the fractional ratio of TOM70 in the TOM complex may support the putative dynamic nature of this protein in the assembled complex.

1·2·3·2 The TOM20-TOM22 receptor subcomplex

Biochemical studies were initially used to elucidate which mitochondrial surface proteins were involved in protein import. Antibodies against TOM20 in *N. crassa* inhibited the import of many preproteins with the exception of the ADP/ATP carrier (AAC) which was only mildly affected (Söllner *et al.*, 1989). Immunoinhibition studies with yeast TOM20 gave similar results (Moczko *et al.*, 1993). Depletion of TOM20 in *N. crassa* results in a number of

deleterious effects including loss of mitochondrial cristae and cytochromes, and a decrease in mitochondrial protein synthesis. These defects contribute to a severe growth defect in mutant strains (Harkness *et al.*, 1994). Preprotein import into isolated mitochondria depleted of TOM20 is strongly reduced for all precursors tested with the exceptions of AAC and a fusion preprotein consisting of the presequence of cytochrome c_1 attached to cytochrome *c*. Yeast cells with a deletion of the gene encoding TOM20 are respiratory deficient, cannot grow on a non-fermentable carbon source, and grow at a reduced rate on fermentable carbon sources (Moczko *et al.*, 1994). Import of preproteins into isolated TOM20-deficient yeast mitochondria was also strongly reduced. In both *N. crassa* and yeast, loss of TOM20 results in reduced levels of TOM22. Antibodies raised against TOM22 inhibit the import of almost all preproteins tested (Kiebler *et al.*, 1993). Genetic analysis demonstrated that TOM22 is an essential component of the TOM complex in both *N. crassa* and yeast (Hönlinger *et al.*, 1995; Nargang *et al.*, 1995). Preprotein import into isolated mitochondria lacking TOM22 is almost completely inhibited in both organisms. Increased levels of TOM22 in yeast can compensate for loss of TOM20 (Lithgow *et al.*, 1994b).

Both TOM20 and TOM22 have been shown to interact with mitochondrial preproteins (Mayer *et al.*, 1995a; Söllner *et al.*, 1992). It has been suggested that TOM20 and TOM22 form a receptor subcomplex where initial binding of the preprotein targeting sequence takes place (Mayer *et al.*, 1995a). This structure has been termed the *cis*-binding site. The exact nature of the *cis*-site has yet to be determined. An attractive model suggests that the negatively charged amino acid residues present in the cytosolic domain of TOM22 interact with the positive charges on the hydrophilic face of the α-helical structure of N-terminal presequences through electrostatic

interactions (Kiebler *et al.*, 1993). However, this does not explain how the TOM20-TOM22 subcomplex also recognizes preproteins without the classical cleavable presequences, such as porin and cytochrome *c* heme lyase. Furthermore, when the negatively charged residues of the TOM22 cytosolic domain in *N. crassa* are neutralized to an extent that leaves the domain with a net positive charge, import of precursors with both cleavable and non-cleavable targeting sequences is not affected (Nargang *et al.*, 1998). This suggests that there are additional recognition elements in *cis*-site binding that have yet to be identified.

1·2·3·3 The TOM70-TOM37 receptor subcomplex

TOM70 was first identified in *N. crassa* as a specific receptor for the ADP/ATP carrier (AAC) (Söllner *et al.*, 1990). Antibodies directed against TOM70 strongly inhibited the binding of AAC on the mitochondrial surface. Cleavage of the cytosolic domain of TOM70 by mild protease treatments also significantly reduced the amount of specifically bound AAC on the mitochondrial surface (Steger *et al.*, 1990). Yeast mitochondria deficient in TOM70 were also shown to have strongly reduced levels of bound AAC (Steger *et al.*, 1990). The import of certain preproteins such as AAC, phosphate carrier, the β-subunit of the F_1-ATPase, and cytochrome c_1 into these mitochondria was shown to be significantly reduced (Hines *et al.*, 1990). TOM70 mutant strains in yeast show a reduction in growth rate on non-fermentable carbon sources, the severity of which is exacerbated by increasing temperature (Riezman *et al.*, 1983). No observable growth defect was seen on glucose-rich medium at 23°C, but growth may be slightly impaired at 37°C (Steger *et al.*, 1990). Direct binding of AAC with TOM70 was found by co-immunoprecipitation and cross-linking experiments (Steger

et al., 1990) (Söllner *et al.*, 1990). *In vitro* experiments using intact mitochondria demonstrated that the purified 65-kDa cytosolic domain of *N. crassa* TOM70 can compete with TOM70 for binding and reduce the import of AAC, and to a lesser degree cytochrome c_1 (Schlossmann *et al.*, 1994). Most other precursors were unaffected. Since import of those preproteins which depend upon TOM70 is not completely abolished by its absence on the mitochondrial surface, these preproteins can alternatively use the TOM20-TOM22 sub-complex for import into mitochondria (Lithgow *et al.*, 1994b; Steger *et al.*, 1990).

TOM71 is a closely related homologue of TOM70 in yeast. The abundance of TOM71 in mitochondria is at least 8-10 fold lower than that of TOM70 (Bömer *et al.*, 1996a). Deletion of the gene that encodes TOM71 results in a slightly more decreased growth rate at 37°C on non-fermentable carbon sources than that produced by the deletion of *tom70* (Schlossmann *et al.*, 1996). A double-deletion of both *tom70* and *tom71* had an indistinguishable growth phenotype from wild-type on glycerol-containing medium at 24°C and 30°C demonstrating that TOM71 is not an essential protein even in the absence of TOM70 (Schlossmann *et al.*, 1996). At 37°C, the growth rate of the double-deletion mutant on glycerol-containing medium showed reduced growth relative to wild-type cells, and was indistinguishable from the *tom71* mutant grown in identical conditions. Double-deletion mutant cells grew at wild-type rates on glucose-containing medium under the same conditions. Isolated mitochondria lacking TOM71 demonstrated a less pronounced, but still significant, decrease in AAC surface binding compared to that of TOM70-deficient mitochondria. AAC surface binding in mitochondria lacking both TOM70 and TOM71 was reduced to background levels. These data suggest that TOM71 can bind AAC, but at a lower affinity

than TOM70. Import of AAC into isolated mitochondria lacking TOM71 was reduced, but import of other preproteins tested showed no significant reductions. The function of TOM71 in relation to TOM70, and the rest of the TOM complex, remains unclear.

TOM37 is a non-essential component of the TOM complex that has thus far only been identified in yeast. Deletion of *tom37* results in mild growth defects similar to those seen in strains with *tom70* and *tom71* deletions (Gratzer *et al.*, 1995). Interestingly, double deletions of *tom37* and either *tom70* or *tom20* are lethal. Antibodies directed against TOM37 inhibited the import of AAC by 70% and that of cytochrome c_1 by 30%. Similarly, import of AAC into isolated mitochondria lacking TOM37 is defective. The precise function of TOM37 has yet to be explained. It is proposed that TOM37 and TOM70 forms a heterodimer that functions as a receptor for a subclass of mitochondrial preproteins represented by the carrier family of proteins. As previously mentioned, mitochondrial import stimulating factor (MSF) in complex with a preprotein appears to be targeted to the TOM70-TOM37 subcomplex. Therefore, this receptor subcomplex may provide a docking site for the MSF-preprotein complex, although direct interaction of TOM37 with preproteins has yet to be demonstrated (Hachiya *et al.*, 1995; Mihara and Omura, 1996).

1·2·3·4 The translocation pore

Once preproteins interact with the surface receptors they are transported across the outer membrane through the general insertion pore. The primary candidate for a pore component is TOM40, an essential protein in *N. crassa* and yeast. TOM40 is deeply embedded in the membrane (Kiebler *et al.*, 1990) and is in contact with preproteins as they cross the

mitochondrial outer membrane (Vestweber *et al.*, 1989). It has been predicted to span the membrane many times in the form of β-strands similar to those of bacterial and mitochondrial outer membrane porins (Court *et al.*, 1995). How TOM40 actually contributes to the structure of the pore has yet to be determined although stoichiometric data suggests that several TOM40 molecules are involved in formation of a translocation channel rather than a single TOM40 constituting the pore (Künkele *et al.*, 1998). TOM40 has also been shown to have a dynamic structure as an increased level of TOM40 dimers exists in the absence of precursor protein (Rapaport *et al.*, 1998a). The ratio of TOM40 dimers to monomers is also influenced by a block of negatively charged amino acid residues in the cytosolic domain of TOM22 indicating a direct relationship between the preprotein receptors on the surface of the outer membrane and the translocation pore. When preproteins are bound to either side of the mitochondrial outer membrane, distinct structural alterations of TOM40 are induced which may be crucial for the movement of preproteins across the membrane.

The smaller components of the TOM complex, TOM5, TOM6, and TOM7 seem to be part of this pore as well. Disruption of TOM5 in yeast results in reduced growth at 30°C on fermentable and non-fermentable carbon sources, and no growth at 37°C (Dietmeier *et al.*, 1997). Preproteins with and without cleavable presequences had reduced rates of import into isolated yeast mitochondria lacking TOM5. TOM5 is closely associated with TOM40 and may represent a functional link between the surface receptors and the general insertion pore. The negatively charged cytosolic domain of TOM5 is thought to position incoming preproteins directly above the import pore prior to translocation. TOM6 and TOM7 do not interact with preproteins in transit but modulate the dynamics of the translocation pore. TOM6 is

anchored in the mitochondrial outer membrane by a C-terminal hydrophobic segment (Cao and Douglas, 1995) and can co-immunoprecipitate with antibodies against TOM40 (Alconada *et al.*, 1995). The protein was shown to promote the association of the two receptor sub-complexes, TOM20-TOM22 and TOM70-TOM37, with TOM40 and accelerated preprotein import into isolated mitochondria by about three-fold. Upon deletion of *tom6*, the stability between the two receptor subcomplexes and TOM40 is decreased, but without changing the total mitochondrial amounts of these TOM proteins. TOM7 appears to function in an antagonistic manner to TOM6 (Hönlinger *et al.*, 1996). The absence of TOM7 in yeast stabilizes the interactions of TOM20, TOM22, and TOM40. Deletion of *tom7* inhibited mitochondrial import of the outer membrane protein porin, but only slightly impaired import for those preproteins destined for interior mitochondrial subcompartments. It is proposed that TOM7 plays a role in sorting and accumulation of preproteins at the outer membrane and also complements the action of TOM6 in modulating the dynamics of the TOM complex.

During or after translocation through the general insertion pore, preproteins are thought to interact with a *trans*-binding site. In this position, the cleavage site of a presequence is exposed to the inner side of the mitochondrial outer membrane as shown by using outer membrane vesicles in which matrix processing peptidase (MPP) is trapped in the lumen (Mayer *et al.*, 1995b). Preproteins bound at the *trans*-site can be cleaved by trapped MPP, but not by extra-vesicular MPP. *Trans*-site intermediates are bound much more firmly than *cis*-site intermediates, and their binding is largely salt-resistant (Rapaport *et al.*, 1998b). The precise location of the *trans*-binding site has yet to be determined due to contradictory results of past investigations. The small negatively charged intermembrane space domain

of TOM22 was initially seen as a likely candidate. Chemically synthesized presequence peptides were found to bind to the purified C-terminus of TOM22 (Bolliger *et al.*, 1995). An investigation in which the intermembrane space domain of yeast TOM22 was deleted showed a moderate reduction in import of preproteins with cleavable N-terminal presequences, but no effect on the import of preproteins with internal targeting information (Moczko *et al.*, 1997). Conversely, in another study involving yeast TOM22 (Nakai *et al.*, 1995), and a similar one involving *N. crassa* TOM22 (Court *et al.*, 1996), deletion of the intermembrane space domain appeared to have only minor or no inhibitory effects on *in vitro* mitochondrial protein import. A more appealing candidate for a *trans*-binding site is TOM40 which also has segments exposed to the intermembrane space. *Trans*-binding of preproteins has been shown to occur at TOM40 (Rapaport *et al.*, 1998b).

1·2·4 Translocases of the mitochondrial inner membrane: the TIM complexes

1·2·4·1 Components of the TIM complexes

The translocation machinery of the mitochondrial inner membrane can function independently of the TOM complex (Hwang *et al.*, 1989; Hwang *et al.*, 1991; Segui-Real *et al.*, 1993). Two distinct TIM complexes, each having its own specialized function have been characterized. Two molecular chaperones that reside in the matrix are also associated with one of the TIM complexes. A list of the known components of the two TIM complexes is presented in Table 1-2. Similar to that for the components of the TOM complex, a uniform nomenclature reflecting consensus molecular weights is used to identify constituents of the TIM complex (Pfanner *et al.*, 1996).

Table 1-2. Components of the translocase of the mitochondrial inner membrane.

Component	Other names	Identified in yeast	Identified in *N. crassa*	Function
TIM23	Mas6p MIM23	Yes	No	Component of the import channel; $\Delta\psi$-dependent binding of presequences (Dekker *et al.*, 1993; Bauer *et al.*, 1996).
TIM17	MIM17 Sms1	Yes	Yes	Component of the import channel (Ryan *et al.*, 1994; Berthold *et al.*, 1995; Maarse *et al.*, 1994; Tan and Nargang, unpublished).
TIM44	Mpi1p ISP45	Yes	No	Associates with import channel; binds mt-Hsp70 in ATP-dependent fashion; helps drive preprotein translocation (Maarse *et al.*, 1992; Schneider *et al.*, 1994; Rassow *et al.*, 1994).
mt-Hsp70	Ssc1	Yes	Yes	DnaK-type chaperone; facilitates import, folding, and assembly of proteins in the matrix; interacts with TIM44 (Schneider *et al.*, 1994; Kronidou *et al.*, 1994).
MGE1	Mge1p mGrpE Yge1p	Yes	No	Co-chaperone; cooperates with mt-Hsp70 (Bolliger *et al.*, 1994; Voos *et al.*, 1994; Westermann *et al.*, 1996).

TIM22		Yes	No	Facilitates import of carrier proteins and other components of the TIM complex into the inner membrane (Sirrenberg *et al.*, 1996; (Sirrenberg *et al.*, 1998).
TIM10	Tim10p Mrs11	Yes	No	Associates with TIM22; may function as inner membrane chaperone for carrier insertion into the inner membrane (Sirrenberg *et al.*, 1998; Koehler *et al.*, 1998).
TIM12	Tim12p Mrs5	Yes	No	As TIM10.
TIM54		Yes	No	Associates with TIM22; required for insertion of proteins into the inner membrane (Kerscher *et al.*, 1997).

Almost all of the investigations done on the components of the TIM complex have been done in yeast.

The first TIM complex identified consists of TIM17, TIM23, and TIM44. This TIM complex is responsible for translocating preproteins across the mitochondrial inner membrane and into the matrix. TIM23 is an integral 23-kDa protein of the yeast mitochondrial inner membrane with a negatively charged hydrophilic N-terminal region and a C-terminal hydrophobic region (Dekker *et al.*, 1993). The hydrophilic region is exposed to the intermembrane space while the hydrophobic domain most likely spans the inner membrane three to four times (Kübrich *et al.*, 1994). TIM17 is another integral membrane protein that has a hydrophobic domain with high sequence similarity to that of the hydrophobic domain of TIM23 (Maarse *et al.*, 1994; Ryan *et al.*, 1994). TIM17 and TIM23 appear to have similar orientations within the inner membrane, but TIM17 lacks a hydrophilic intermembrane space domain. Homologues of TIM17 have been found in the sequence databases of *Homo sapiens*, *Drosophila melanogaster*, *Caenorhabditis elegans*, and *Arabidopsis thaliana*, demonstrating that the protein is highly conserved throughout evolution (Bömer *et al.*, 1996b). In contrast, TIM44 is a hydrophobic protein with a predicted membrane-spanning domain (Maarse *et al.*, 1992), and is associated peripherally to the matrix side of the mitochondrial inner membrane. It is clear that the majority of the protein is exposed to the matrix side of the inner membrane. TIM23 and TIM17 form a complex with about 1:1 stoichiometry (Blom *et al.*, 1995) and TIM44 has been shown to associate with the TIM23-TIM17 subcomplex (Berthold *et al.*, 1995).

The second TIM complex identified contains TIM22, a hydrophobic inner membrane protein with homology to both TIM23 and TIM17. This TIM complex is responsible for inserting preproteins into the mitochondrial inner

membrane. TIM22 does not associate with either TIM23 or TIM17 (Sirrenberg *et al.*, 1996). TIM54, an integral inner membrane protein with its C-terminus exposed to the intermembrane space, has been shown to co-precipitate with TIM22 in yeast (Kerscher *et al.*, 1997). TIM10 and TIM12, two cysteine-rich hydrophilic proteins that are found in the intermembrane space form a complex with TIM22 (Koehler *et al.*, 1998; Sirrenberg *et al.*, 1998). An 11 kDa inner membrane protein, termed TIM11, was originally identified as a component of the protein import system in the inner membrane (Tokatlidis *et al.*, 1996). This protein was later identified as a novel subunit of the yeast mitochondria F_1F_0-ATPase (Arnold *et al.*, 1997). Its function in mitochondria protein import is not well defined.

1·2·4·2 Import of preproteins into the matrix

TIM17 and TIM23 are the most likely components of an inner membrane translocation pore through which preproteins can enter the matrix, since both have been cross-linked to preproteins in transit to the matrix (Berthold *et al.*, 1995; Ryan and Jensen, 1993). It is unclear whether TIM44 also contributes to this pore. All three components are essential in yeast. The hydrophilic N-terminal domain of TIM23, which contains a net negative charge, has the ability to bind presequence peptides and may provide a *cis*-binding site for preproteins being translocated through the inner membrane (Emtage and Jensen, 1993). TIM23 forms a dimer in the presence of a membrane potential ($\Delta\Psi$) most likely via a leucine repeat motif with a predicted potential for dimerization (Bauer *et al.*, 1996). These observations led to the suggestion that TIM23 may act as a membrane potential ($\Delta\Psi$)-dependent translocation channel. $\Delta\Psi$ has been shown to be essential for preprotein translocation across the mitochondrial inner membrane (Pfanner

and Neupert, 1985). Its purpose may be two-fold, not only regulating the opening and closing of the TIM23 voltage-dependent translocation channel, but it may also exert an electrophoretic force on positively charged presequences leading to inward movement into the matrix.

TIM44 has been shown to closely associate with preproteins in transit to the matrix (Blom *et al.*, 1993), but is most likely not part of the actual translocation pore. In the absence of preproteins, TIM44 forms a stable TIM44-TIM23 subcomplex (Bömer *et al.*, 1997). A key function of TIM44 is its ability to reversibly bind mitochondrial Hsp70 (mtHsp70; (Kronidou *et al.*, 1994)). mtHsp70 is essential to mitochondrial preprotein import and maintaining incoming preproteins in a linear conformation once in the matrix (Kang *et al.*, 1990). It has been found to interact with preproteins in transit (Gambill *et al.*, 1993; Scherer *et al.*, 1990). It has been proposed that the interaction of mtHsp70 with incoming preproteins drives the translocation of polypeptide chains into the matrix via a dynamic interaction with TIM44. When a polypeptide chain emerges from the inner membrane translocation pore into the matrix, it interacts with TIM44 which recruits mtHsp70 (Kronidou *et al.*, 1994; Rassow *et al.*, 1994; Schneider *et al.*, 1994). The interaction of mtHsp70 with TIM44 triggers a transfer of the incoming polypeptide chain from TIM44 to mtHsp70. Progressive cycles of binding and release of mtHsp70 with the preprotein is dependent upon the hydrolysis of ATP. There are presently two models used to describe the mechanism of translocation into the matrix (Glick, 1995). The 'molecular rachet' model states that a translocated polypeptide chain can passively diffuse across inner membrane import channels, which progressively exposes more C-terminal segments to TIM44 (Schneider *et al.*, 1994). mtHsp70 then associates with the TIM44-preprotein complex, which prevents the polypeptide from slipping back

through the TIM translocation pore. Continual cycles of mtHsp70 binding to the preprotein complex is thought to drive vectorial translocation into the matrix. Another model has mtHsp70 performing a more active role in translocation (Pfanner and Meijer, 1995) by exerting a 'pulling' force on a bound preprotein via conformational changes induced by cycles of ATP hydrolysis. This conformational change generates a mechanical force that pulls a preprotein into the matrix. Conceivably, a combination of the two models may be used in preprotein translocation into the matrix (Voos *et al.*, 1996).

1·2·4·3 Matrix components

Once preproteins are translocated into the matrix, the N-terminal presequence extension is proteolytically cleaved by a dimeric matrix protease, the matrix processing peptidase (MPP; Brunner *et al.*, 1994). In addition, a number of molecular chaperones in the matrix can interact with incoming polypeptides. As mentioned above, mtHsp70 is needed for translocation. MDJ1, the mitochondrial homologue of bacterial DnaJ, interacts with preproteins and cooperates with mtHsp70 as a co-chaperone (Westermann *et al.*, 1996) and may be used to keep preproteins in an unfolded state during translocation in order to prevent misfolding and aggregation (Hartl *et al.*, 1994). The matrix protein MGE1, the eukaryotic homologue of the bacterial GrpE protein has also been shown to play an essential role in translocation (Laloraya *et al.*, 1995; Westermann *et al.*, 1995). The protein is thought to exchange mtHsp70-bound ADP for ATP. Mutations in MGE1 cause accumulation of mtHsp70-ADP which associates with TIM44 in a non-productive form thereby inhibiting preprotein import into the matrix (Westermann *et al.*, 1995). Sometime during or after proteolytic

cleavage, imported proteins fold back to their native conformation. A number of matrix chaperones can facilitate this protein folding. Hsp60, a chaperonin that forms a 14-mer and requires ATP-hydrolysis (Höhfeld and Hartl, 1994; Martin *et al.*, 1991) mediates protein folding. Hsp60 requires regulation by another chaperonin, Hsp10, that forms a heptamer. A protein released from mtHsp70 interacts with the Hsp60/Hsp10 complex. A folding cage is formed by the Hsp60 14-mer, and covered by the Hsp10 heptamer, which encloses an unfolded protein in its central cavity and promotes folding of the protein (Cheng *et al.*, 1989; Ostermann *et al.*, 1989). This requires several cycles of ATP-hydrolysis. Other components known to facilitate protein folding in the matrix include mitochondrial cyclophilins (Matouschek *et al.*, 1995; Rassow *et al.*, 1995).

1·2·4·4 Sorting of preproteins to the inner membrane

TIM22 was identified as an essential protein in yeast that exists in a protein complex in the mitochondrial inner membrane distinct from that of the TIM17-TIM23-TIM44 complex (Sirrenberg *et al.*, 1996). Depletion of TIM22 does not affect preprotein import into the matrix, but does inhibit insertion of hydrophobic preproteins into the inner membrane. TIM22 is also required to insert TIM17 and TIM23 into the inner membrane (Sirrenberg *et al.*, 1996). Insertion of preproteins into the inner membrane also requires a membrane potential. TIM54, an essential protein in yeast, is required for insertion of at least two polytopic proteins into the inner membrane and was shown to co-immunoprecipitate with TIM22 (Kerscher *et al.*, 1997). Mutations in TIM54 show destabilizing effects on TIM22, but not on TIM23 or TIM17. TIM10 and TIM12 are found in a complex with TIM22 and are both essential for viability in yeast (Koehler *et al.*, 1998; Sirrenberg *et al.*, 1998). Mutation in either

protein showed defects in insertion of carrier proteins, but not for preproteins targeted to other mitochondrial sub-compartments. TIM10 and TIM12 are also required for insertion of TIM22 into the inner membrane. Both proteins contain a zinc-finger-like motif with four cysteines and their interaction with preproteins depends on the presence of divalent metal ions. This zinc finger motif is predicted to complement the pattern of charged and hydrophobic amino acid residues present in the 'carrier motif' found in inner membrane carrier proteins (Sirrenberg *et al.*, 1998). TIM10 and TIM12 may function as intermembrane space chaperones for the hydrophobic carrier family of proteins.

1·3 Objectives of this study

The purpose of this study was to investigate the functional role of the mitochondrial outer membrane protein TOM70 in *N. crassa* using a combination of genetic and biochemical approaches. There are a number of reasons for doing so. First, only biochemical techniques have been used to study the functional role of *N. crassa* TOM70 in mitochondrial preprotein import. Past investigations have used antibodies against TOM70 (Söllner *et al.*, 1990), and specific proteolytic cleavage of the cytosolic domain of TOM70 to determine which preproteins were inhibited from binding to the mitochondrial surface (Steger *et al.*, 1990). These studies demonstrated a significant decrease in the amount of ADP/ATP carrier (AAC) bound to the surface of isolated mitochondria. There are inherent problems with using these approaches. The use of proteases is not specific since other surface receptors involved in preprotein import may also be inadvertently cleaved. The use of antibodies to inhibit binding of preproteins to TOM70 may sterically hinder other precursors from binding other components of the TOM

complex in the vicinity of TOM70. More importantly, in both techniques the protein, or some part of it, is still present within the mitochondrial outer membrane. The presence of any segment of TOM70 in the outer membrane may not fully demonstrate how the absence of the protein affects the assembly of the TOM complex.

Second, the effects of inactivating the *tom70* gene in *N.crassa* have yet to be observed. The complete absence of TOM70 within the organism would be the ideal approach to study its putative function within the cell. Genetic approaches have been used to analyze TOM70 in yeast. Initially, it was found that the absence of yeast TOM70 inhibited the preprotein import of AAC, and F_1-ATPase α- and β-subunits (Hines *et al.*, 1990). More recent investigations have shown that carrier proteins, and to a lesser degree cytochrome c_1, appear to be dependent upon yeast TOM70 for efficient import into isolated mitochondria (Schlossmann and Neupert, 1995), while most other preproteins were not affected. *N. crassa* TOM70 shares only 33.0% amino acid sequence identity and 45.9% protein similarity with yeast TOM70 (Steger *et al.*, 1990), therefore there may be inherent differences in the function and mode of action of TOM70 in the two organisms. In support of this the *N. crassa* protein cannot substitute for the yeast homologue (Schlossmann and Neupert, 1995), and two components closely associated with TOM70 in yeast, TOM37 and TOM71, have yet to be identified in purified preparations of the *N. crassa* TOM complex (Künkele *et al.*, 1998).

Third, only a fraction of the total *N. crassa* TOM70 is found in complex with the other TOM components (Söllner *et al.*, 1992). This may indicate that TOM70 has a secondary function other than that of a preprotein receptor. The seven tetratricopeptide repeat (TPR) motifs present in TOM70 may indicate a putative role in interacting with cytoskeletal factors (Söllner *et al.*,

1990) since other proteins within the TPR gene family include those required for mitosis, a process closely associated with the cytoskeleton (Goebl and Yanagida, 1991). For these reasons, *N. crassa* TOM70 was investigated by inactivating the *tom70* gene using the natural phenomenon of repeat induced point mutation (RIP). Mutants that lacked TOM70 were studied to determine the functional role of the protein within the cell.

1·4 References

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Chapter 2: Inactivation of the *Neurospora crassa* gene encoding the mitochondrial outer membrane protein TOM70 using the phenomenon of RIP

2·1 Introduction

In *Saccharomyces cerevisiae*, the method most commonly used to inactivate target genes is to replace the endogenous copy of the gene with another copy of the gene disrupted by a large sequence of DNA, usually containing a selectable marker, by homologous integration (Rothstein, 1991). While this method is effective in yeast, it has achieved limited success in *N. crassa* (Frederick *et al.*, 1989; Nehls *et al.*, 1992) most likely because the frequency of replacement by homologous integration appears to be very low for many *N. crassa* genes (Asch and Kinsey, 1990). An efficient alternative to gene disruption is to make use of a natural phenomenon known as repeat induced point mutation (RIP) which has been described in *N. crassa* (Selker, 1990) and a few other filamentous fungi. The process of RIP occurs only during the sexual cycle and affects duplicated sequences of DNA present in the nucleus by effectively mutating both copies with a variable number of GC to AT transitions. RIP is thought to be an internal mechanism by which the cell streamlines the genome by limiting the amount of selfish DNA. In support of this, the genome of *N. crassa* carries very little in the way of repetitive sequences.

RIP can be utilized in the laboratory by transforming a *N. crassa* strain to create a duplication of a target gene that will subsequently be mutated when the strain is taken through a sexual cross. This approach has been successfully used to create viable mutants for a number of non-essential *N. crassa* genes in past investigations (Barbato *et al.*, 1996; da Silva *et al.*, 1996;

Seiler *et al.*, 1997). Studies in yeast demonstrated that the *tom70* gene is not essential for cell viability (Hines *et al.*, 1990; Steger *et al.*, 1990) and suggested that direct inactivation of *tom70* by RIP could be employed to generate viable mutants for further investigation. This chapter will describe both the genetic details of the RIP procedure as applied to *tom70* and the genetic characteristics of the mutant alleles obtained.

2·2 Materials and Methods

2·2·1 Strains and media

Growth and handling of *N. crassa* strains was as described in Davis and De Serres (1970). All strains used in this study are listed in Table 2-1. Minimal medium consisted of Vogel's salts, trace elements, biotin, and 1.5% sucrose. When required, nicotinamide (10 μg/ml) and D-pantothenic acid (10 μg/ ml) were added to the medium. When required, the inhibitors bleomycin or hygromycin were present in the media at concentrations of 0.17 μg/ml (viability plates) to 0.44 μg/ml (top agar) and 2.5 μg/ml (for viability plates and top agar), respectively.

2·2·2 Plasmid construction

A cDNA version of the *N. crassa tom70* gene was previously cloned (Steger *et al.*, 1990). A PCR-derived product with *Hind*III ends was produced from the cloned cDNA using primers 72T and 72B (Table 2-2). The *tom70* fragment in p72PCR-7 totals 1927 base pairs (bp) and extends 29 bp upstream from the *tom70* start codon and 27 bp downstream from the stop codon. In experiments designed to generate RIP mutations in specific target genes, it is desirable to limit the amount of non-*tom70* DNA in the construct to prevent possible RIP of neighboring genes (Selker, 1990). The *tom70*

Table 2-1. *N. crassa* strains used in this study.

Strain	Genotype
NCN10	*A nic-1 al-2.*
NCN251	A.
NCN235	*a pan-2.*
NCN7 (Emerson)	*A* (wild-type laboratory strain).
NCN20	*A* (wild-type laboratory strain).
NCN27 (Mauriceville-1c)	*A* (wild-type natural strain).
NCN39 (Lein 7)	*A* (wild-type laboratory strain).
ST-2-1	Isolate from transformation of NCN251 with p72PCR-7 (see Fig. 2-1); contains a single ectopically integrated copy of *tom70*; *A* hyg^R.
SU-1-1	Isolate from transformation of NCN10 with p72PCR-7 (see Fig. 2-1); contains a single ectopically integrated copy of *tom70*; *A nic-1 al-2* hyg^R.
SU25	Ascospore isolate of NCN235 and SU-1-1 cross; contains two RIPed alleles of *tom70*; *pan-2* hyg^R.
ST-249	Ascospore isolate of NCN235 and ST-2-1 cross; contains a single RIPed allele of *tom70*; *pan-2*; hyg^S.
SU25-10-6-1	Isolate from transformation of SU25 with pLGR10 (contains *tom70* cDNA; see Fig. 2-2); displayed rescued growth and conidiation phenotypes.
SU25-10-11-1	As SU25-10-6-1.
SU25-10-2-1	As SU25-10-6-1; except displayed unrescued phenotype.
SU25-10-8-1	As SU25-10-2-1.
SU25-B-3-1	As SU25-10-2-1, except that transformation was with a plasmid that did not contain a copy of *tom70* cDNA.

ST-249-10-1-1	Isolate from transformation of ST-249 with pLGR10 (see Fig. 2-1); displayed normal growth rate, but poor conidiation.
ST-249-10-2-1	As ST-249-10-1-1, except displayed normal growth and conidiation phenotypes.
ST-249-10-4-1	As ST-249-10-1-1, except displayed normal conidiation, but poor growth rate.
ST-249-B-1-1	As ST-249-10-1-1, except that transformation was with a plasmid that did not contain a copy of *tom70* cDNA, and displayed unrescued phenotype.

Table 2-2. Primers used for PCR and sequencing of *tom70* alleles.

Primer	Use	Sequence (5' to 3')
72T	PCR/sequencing	CGCCAAGCTTCTCCTACACCCATCACCACA *Hind*III 50 71
72B	PCR/sequencing	CGAGAAGCTTGCTGCTAGAGTAATCTTGAC *Hind*III 2215 2194
LGR2	Sequencing	TTCCAGGAAGACATTGAGAAG 1725 1744
LGR4	Sequencing	CGCTGCATGAAGAACTTCGACCAGA 1649 1673
LGR5	Sequencing	GAGCTGCCCGAGATCGATGAGGAAT 540 564
LGR6	Sequencing	GATGGACGAGTCGGCCGGGACAG 157 135
LGR7	Sequencing	CAAGGGATCAGAGCAGAAGGAGTC 262 intron 1 (118 bp) 403
LGR8	Sequencing	GCTTGCCACAATGCCCTCGCCCAG 729 749
LGR9	Sequencing	GAGAACAAGGCCAAGCAAATCCTCG 954 978
LGR10	Sequencing	CGCCGCCTTCAAGAAGGCCCTCGAC 1166 1190
LGR11	Sequencing	GAGGACTTCAACAAGGCCATTGAG 1433 1456
LGR13	PCR	GGCTGCAGGAATTCGATATCAAG vector sequence upstream of 5' end of *tom70* ectopic copy
LGR14	PCR	GAATGCACAGGTACACTTGTTTAG vector sequence downstream of 3' end of *tom70* ectopic copy

LGR15	PCR	GACTCCGATCTAGTATATACCCTG	
		2	25
LGR16	PCR/sequencing	CAATCTCCTTACACCGTTACAGC	
		2339	2317
LGR17	Sequencing	CTCGGAGCACAAGGCTGTTGTCTAC	
		181	205
LGR18	Sequencing	ATCTCAGCAAGTCCATCGAGCTCGA	
		1281	1306
LGR19	Sequencing	TAAGGCAGTGGAAAGTACCACAGAG	
		1254	1230
FNA153	Sequencing	GAACTTGAGGGCCTCGACGACCT	
		2132	2110

The numbers below each sequence correspond to the numbering system in Fig. 2-6 and indicate the location and orientation of the primer with respect to the *tom70* gene. Underlined sequences indicate an added restriction site. The CGCC and CGAG sequences in 72T and 72B, respectively, were included prior to the restriction site to facilitate digestion with the desired enzyme.

fragment was inserted into plasmid pCSN43 (Staben *et al.*, 1989) to yield p72PCR-7 (Fig. 2-1). This plasmid was used to generate an ectopic duplication of *tom70* in a normal laboratory strain for use in a RIP cross. pCSN43 contains a bacterial *hph* (hygromycin B phosphotransferase) gene expressed by a *trpC* promoter from *Aspergillus nidulans*. Resistance to hygromycin allows for selection of transformants in *N. crassa*.

The *tom70* cDNA clone (Steger *et al.*, 1990) was also utilized to construct the plasmid pLGR10 (Fig. 2-2), which contains a 2.2 kb DNA sequence representing the entire *tom70* cDNA cloned into the *EcoR*I restriction site of pGEM-4 (Steger *et al.*, 1990). pLGR10 also carries a bleomycin-resistance (*ble*) gene. The *ble* cassette was removed from the *N. crassa* transformation vector pAB520 (Austin *et al.*, 1990) by cutting with *BamH*I and *Hind*III. This fragment was cloned into the multiple cloning site of pGEM-4 already containing the *tom70* cDNA. Resistance to bleomycin provides a selectable marker for *N. crassa* transformation. pLGR10 was used to rescue *N. crassa* strains containing genetically inactivated *TOM70* genes.

2·2·3 Isolation of mitochondria

Mycelia from strains NCN235 and SU25 were grown in liquid media for 16 and 20 h, respectively, and harvested by filtration. Mitochondria were isolated as described in Pfanner and Neupert (1985; also see Section 3·2·3). Mitochondrial protein was quantified using the Bio-Rad protein assay (Hercules, CA).

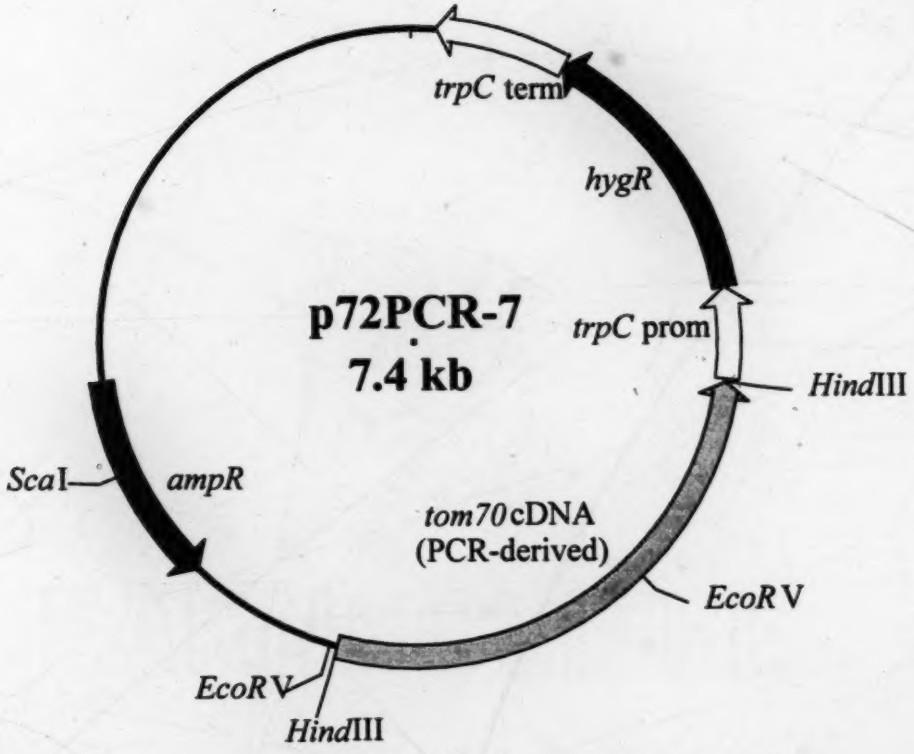


Figure. 2-1. Plasmid p72PCR-7. This plasmid was used to generate an ectopic duplication of *tom70* in a normal strain of *N. crassa*. Names of genes are indicated inside the circle. The filled arrows indicate the extent and orientation of coding sequences of the hygromycin (*hygR*) and ampicillin (*ampR*) selectable markers. The open arrows indicate the promoter and terminator regions derived from *Aspergillus nidulans*. The gray arrow indicates extent and orientation of the PCR-derived *tom70* cDNA sequence. Restriction sites are outside the circle. The *tom70* sequence was inserted into the plasmid at the indicated *Hind*III sites.

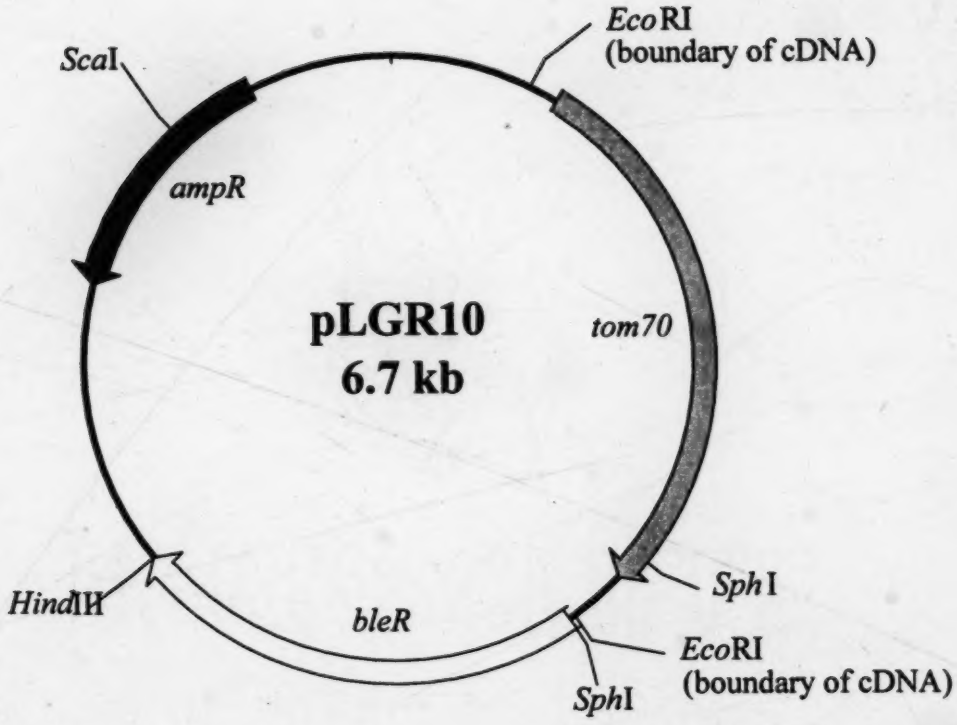


Figure. 2-2. Plasmid pLGR10. This plasmid was used to rescue *tom70*^{RIP} strains. Names of genes are indicated inside the circle. The filled arrow indicates the extent and orientation of the ampicillin (*ampR*) selectable marker. The open arrow indicates extent and orientation of the bleomycin (*bleR*) selectable marker. The gray arrow indicates the extent and orientation of the coding region of *tom70* cDNA. The positions that correspond to the extent of the *tom70* cDNA are indicated by the *Eco*RI sites. Restriction sites are indicated outside of the circle.

2·2·4 Other techniques

The standard techniques of agarose gel electrophoresis, Southern blotting of agarose gels, northern blotting of agarose gels, transformation of *E. coli*, isolation of bacterial plasmid DNA, and the polymerase chain reaction (PCR) using a mixture of *Taq* and *Vent* polymerase (New England Biolabs, Beverly, MA) to minimize replication errors were performed as described in Ausubel *et al.* (1992). The following procedures were also performed in this study using previously published procedures: labeling of DNA Southern probes with digoxigenin-dUTP (DIG) and DIG-labeled nucleic acid colour detection (Boehringer Mannheim, Indianapolis, IN, product information); isolation of total RNA using the Rneasy Plant Total RNA Kit (Qiagen, Chatsworth, CA, product information); separation of mitochondrial proteins by polyacrylamide gel electrophoresis (Laemmli, 1970); western blotting (Good and Crosby, 1989); western blot detection by LumiGLO chemiluminescent substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD, product information); genomic DNA extraction from filamentous fungi (Wendland *et al.*, 1996); transformation of *N. crassa* spheroplasts (Schweizer *et al.*, 1981) with modifications of (Akins and Lambowitz, 1985); and DNA sequencing using Thermosequenase (Amersham, Cleveland, OH, product information). Various individuals in the laboratory of W. Neupert generously supplied antibodies to a number of mitochondrial proteins.

2·3 Results

2·3·1 Generation of tom70^{RIP} mutant strains

In order to inactivate the *tom70* gene by RIP, a strain containing a duplication of *tom70* was to be generated. This initial aspect of the project was conducted by B. Crowther and F. Nargang. Two wild-type laboratory

strains, NCN10 and NCN251, were transformed with p72PCR-7, a hygromycin-resistance plasmid containing a copy of the complete *tom70* coding sequence derived from a cDNA clone by PCR (Fig. 2-1). Transformants were selected on media containing hygromycin. Twenty-five hygromycin-resistant colonies from each transformation were purified through two rounds of single colony isolation on hygromycin medium to ensure that transformants were homokaryotic. NCN251 transformants were referred to as the ST series while NCN10 transformants were called the SU series. Genomic DNA was purified from eighteen isolates from each transformation and examined for the presence of a single ectopically integrated copy of *tom70* derived from p72PCR-7. The *tom70*-specific sequence portion of p72PCR-7 was used to probe Southern blots of *Pst*I-digested genomic DNA isolated from the transformants. Since *Pst*I cuts once within the *tom70* sequence, appropriate transformants containing a single ectopically integrated *tom70* sequence should contain four bands on Southern blots. Two bands should represent the resident, or endogenous copy of *tom70*, while two more bands should represent the ectopically integrated copy. The sizes of the bands of the ectopic copy cannot be predicted and depends upon the position of flanking *Pst*I sites. Six strains were identified that contained single ectopically integrated copies of *tom70*: ST-2-1, ST-6-1, ST-10-1, ST-12-1, SU-1-1, and SU-17-1 (not shown). Four of these strains, ST-2-1, ST-6-1, ST-12-1, and SU-1-1, were crossed with a laboratory wild-type strain of opposite mating type containing only a single copy of *tom70*, NCN235.

At this point I took over the project with the initial goal of identifying those progeny from the cross with RIPed *tom70* genes. RIP occurs after fertilization but before pre-meiotic DNA synthesis during the *N. crassa* sexual cycle (Selker, 1990). Depending on the efficiency of RIP, a substantial

fraction of the progeny should contain *tom70* alleles that underwent RIP. Only a fraction of these will contain *tom70* alleles that have been RIPed severely enough to produce many alterations in the primary protein sequence, or to create a premature stop codon. Individual ascospore isolates from each cross were examined for the presence of TOM70. Mitochondria were isolated from liquid cultures of 78 isolates and examined on western blots using polyclonal antibodies against the entire 65 kDa cytosolic domain of the *N. crassa* TOM70 protein. Two isolates lacking immunologically detectable TOM70, SU25 and ST-249, were identified (Fig. 2-3). A full-length western blot membrane containing mitochondrial protein from both isolates was probed with polyclonal antibodies against TOM70 and revealed no smaller bands that might represent truncated forms of TOM70 (not shown). Both isolates were analyzed further to confirm that the absence of immunologically detectable TOM70 was due to RIP mutagenesis in the target gene.

2·3·2 *Characterization of tom70^{RIP} mutants*

2·3·2·1 Determining copy number of *tom70^{RIP}* alleles

The copy of *tom70* introduced to create the RIP substrate duplication can randomly integrate into any one of the seven chromosomes of *N. crassa*. Therefore, random segregation of chromosomes during meiosis of the RIP cross can result in ascospores that can contain only the endogenous copy of the target gene or both the endogenous and the ectopically integrated copy of the gene. Determining the copy number of *tom70* alleles present in potential RIP mutant strains is important because all copies of *tom70* present in the nucleus must be analyzed to make sure that they are sufficiently RIPed to result in non-functional gene products. To determine how many copies of the

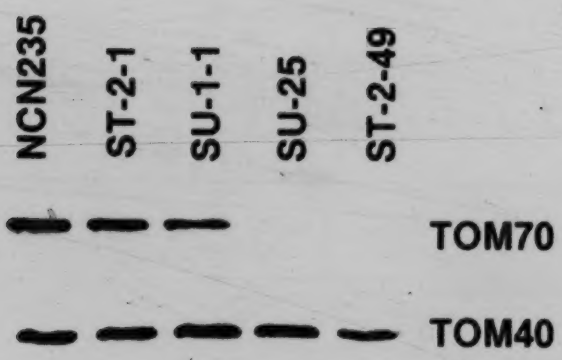


Figure 2-3. Western blot analysis of RIP strains. 15 μg of protein from isolated mitochondria were loaded per lane. Following electrophoresis, proteins were electroblotted to nitrocellulose membrane and probed with polyclonal antibodies to TOM70 and TOM40 (control).

tom70 allele were present in strains SU25 and ST-249, a *tom70*-specific sequence derived from p72PCR-7 was used to probe a Southern blot of *Hind*III-digested genomic DNA isolated from both potential *tom70* mutants. *Hind*III does not cut within the *tom70* gene. Therefore, when probed with a *tom70*-specific probe, strains with only the endogenous copy of the *tom70* gene should contain one band on a Southern blot, while strains with a single ectopic copy of *tom70* in addition to the endogenous copy will contain two bands. Since the PCR-derived 1.9 kb *tom70* cDNA sequence from p72PCR-7 was cloned into the *Hind*III site of the plasmid, the size of the band representing the ectopic copy of *tom70* was also expected to be 1.9 kb, assuming the sites of integration were outside the *tom70* sequence. The results of the Southern blot showed that strain SU25 contained a duplication of *tom70* while strain ST-249 contained only the endogenous copy of the gene (Fig. 2-4).

2·3·2·2 DNA sequence analysis of genomic *tom70*

Sequence analysis of various *tom70* alleles was begun by determining the full genomic sequence of the *tom70* gene since only the cDNA sequence had been previously published (Steger *et al.*, 1990). A pair of PCR primers was designed to amplify the genomic version of *tom70* (primers LGR15 and LGR16; Table 2-2). *tom70* DNA was amplified from genomic DNA of strain NCN235, one of the parental strains involved in both RIP crosses. The relative positions and orientations of all sequencing and PCR primers used in this study are presented in Fig. 2-5. The complete nucleotide sequence of the genomic *tom70* gene is shown in Fig. 2-6. Analysis of the sequence showed that the gene contains four exons separated by three introns of 118 bp, 53 bp, and 63 bp in length. The position of the introns with respect to the

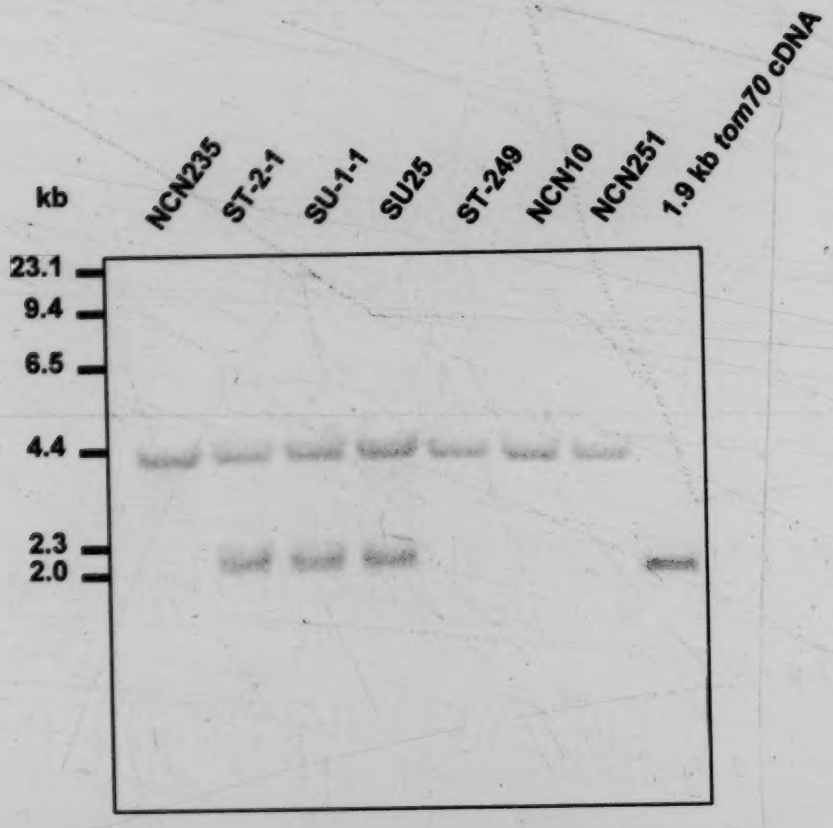


Figure 2-4. Southern blot analysis of *tom70*^{RIP} strains and controls. 10 μg of genomic DNA from indicated strains were digested with the restriction enzyme *Hind*III. Following electrophoresis, DNAs were blotted onto nylon membrane and probed with a DIG-labeled 1.9 kb *Hind*III *tom70* cDNA fragment isolated from p72PCR-7 (Fig. 2-1).

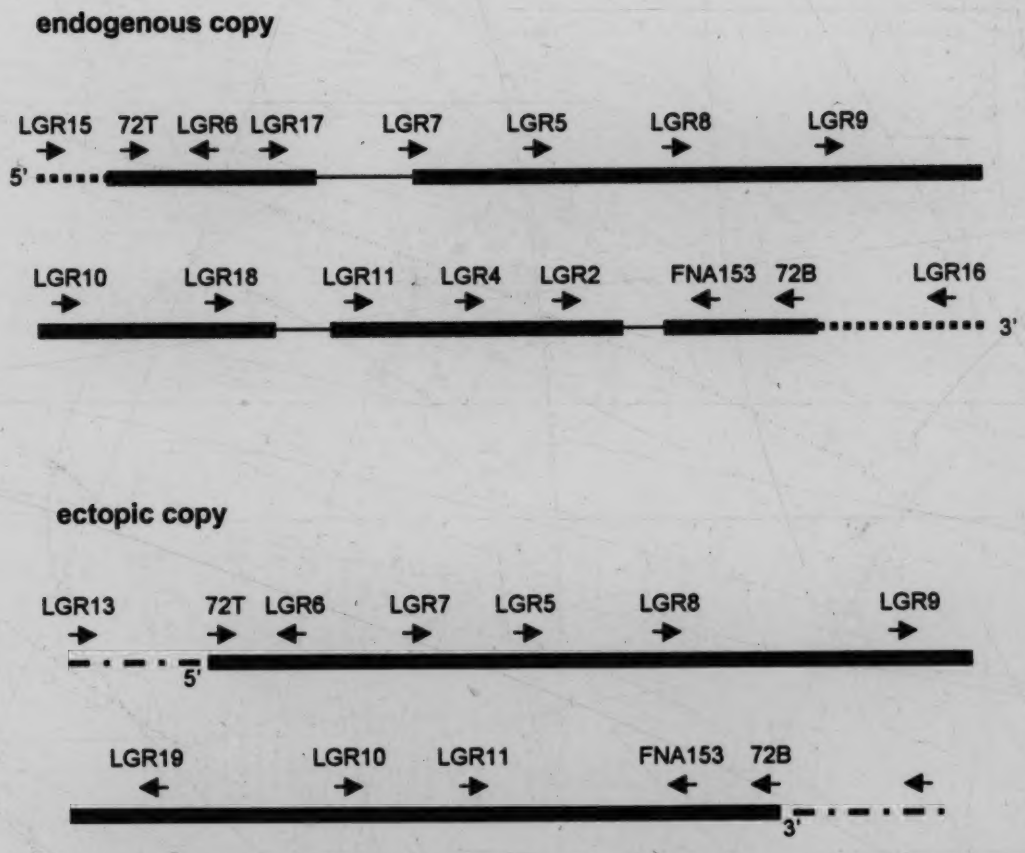
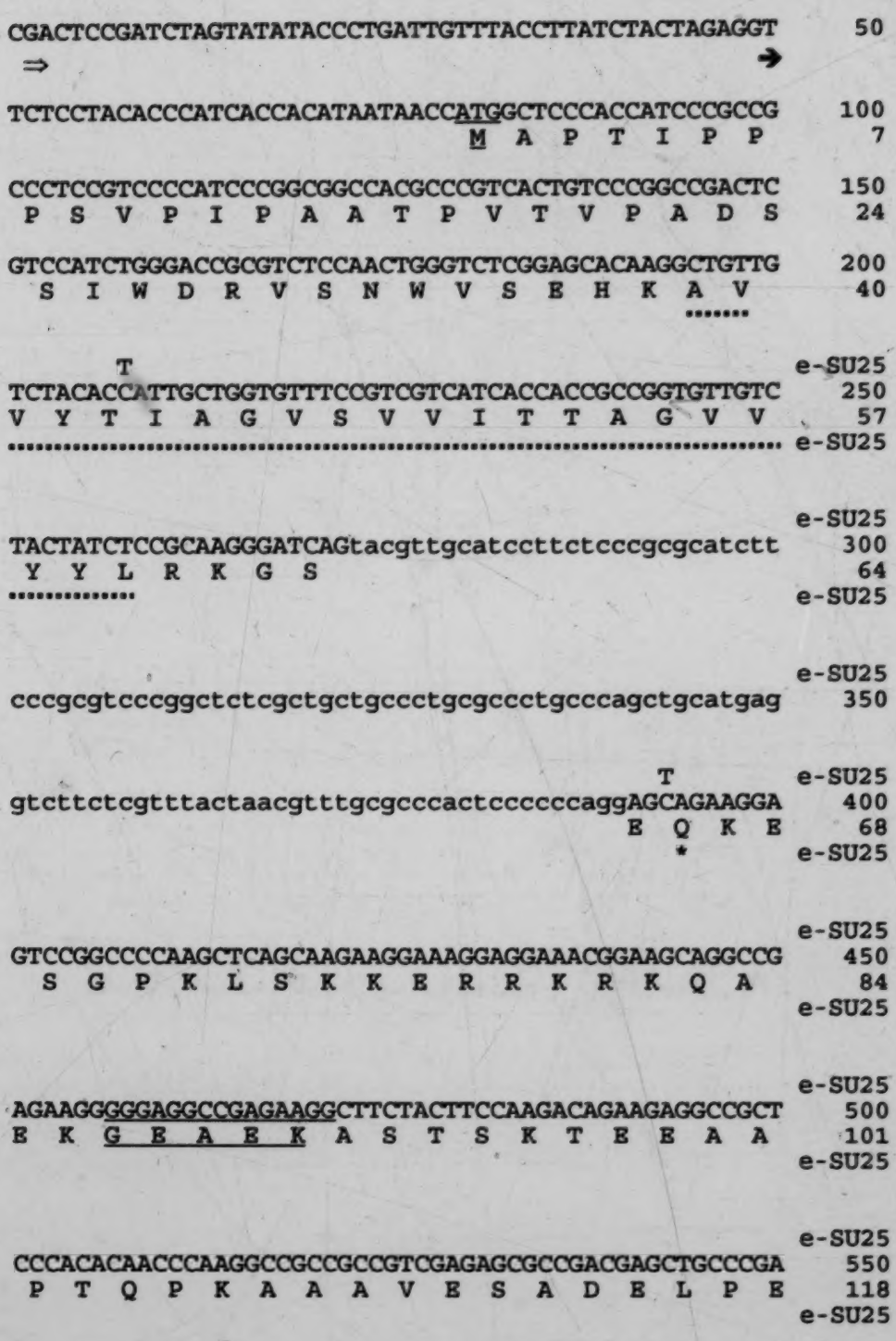


Figure 2-5. Locations of sequencing and PCR primers on schematic digrams of the endogenous and ectopic copies of *tom70*. Arrows indicate positions and orientations of primer with respect to the gene. (.....), 5' and 3' untranslated regions; (▬▬) *tom70* coding sequence; (———), intron regions; (▬ . ▬), vector sequence surrounding ectopically integrated copy of *tom70*. The LGR13 and LGR14 primers were used only for the amplification of the ectopic copy of *tom70*.

Figure 2-6. DNA sequence and predicted amino acid sequence of *tom70* in strain NCN235. The initiation methionine and corresponding start codon are underlined. Bases above the wild-type DNA sequence indicate positions where mutations were found in the three different RIPed alleles of *tom70*. Letters below the wild-type amino acid sequence indicate positions of amino acid changes resulting from RIPs. Small filled arrows below the DNA sequence indicate the extent of sequencing for the RIPed ectopic copy of *tom70* in strain SU25. Small open arrows indicate the extent of sequencing for both the RIPed and wild-type versions of the endogenous copy of *tom70*. Lower-case letters indicate intron sequences. Dashed line underneath amino acid letters indicate the predicted membrane-spanning domain. Amino acid sequences highlighted with gray bars indicate putative tetratricopeptide repeat (TPR) motifs. r-SU25, endogenous copy of *tom70* in strain SU25; e-SU25, ectopic copy of *tom70* in strain SU25, ST-249, endogenous copy of *tom70* in strain ST-249. Asterisks below the amino acid sequence indicate the first premature stop codon occurring in each of the RIPed alleles. Dashes below the amino acid sequence indicate other premature stop codons. Solid line underneath DNA sequence and corresponding amino acid sequence indicate the additional sequence not present in the published version of the *tom70* cDNA (Steger *et al.*, 1990).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⇒ ➔
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M A P T I P P 7
CCCTCCGTCCCCATCCCGGCGGCCACGCCCGTCACTGTCCCGGCCGACTC 150
P S V P I P A A T P V T V P A D S 24
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S I W D R V S N W V S E H K A V 40
T e-SU25
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V Y T I A G V S V V I T T A G V V 57
e-SU25
e-SU25
TACTATCTCCGCAAGGGATCAGtacgttgcatccttctcccgcgcatctt 300
Y Y L R K G S 64
e-SU25
e-SU25
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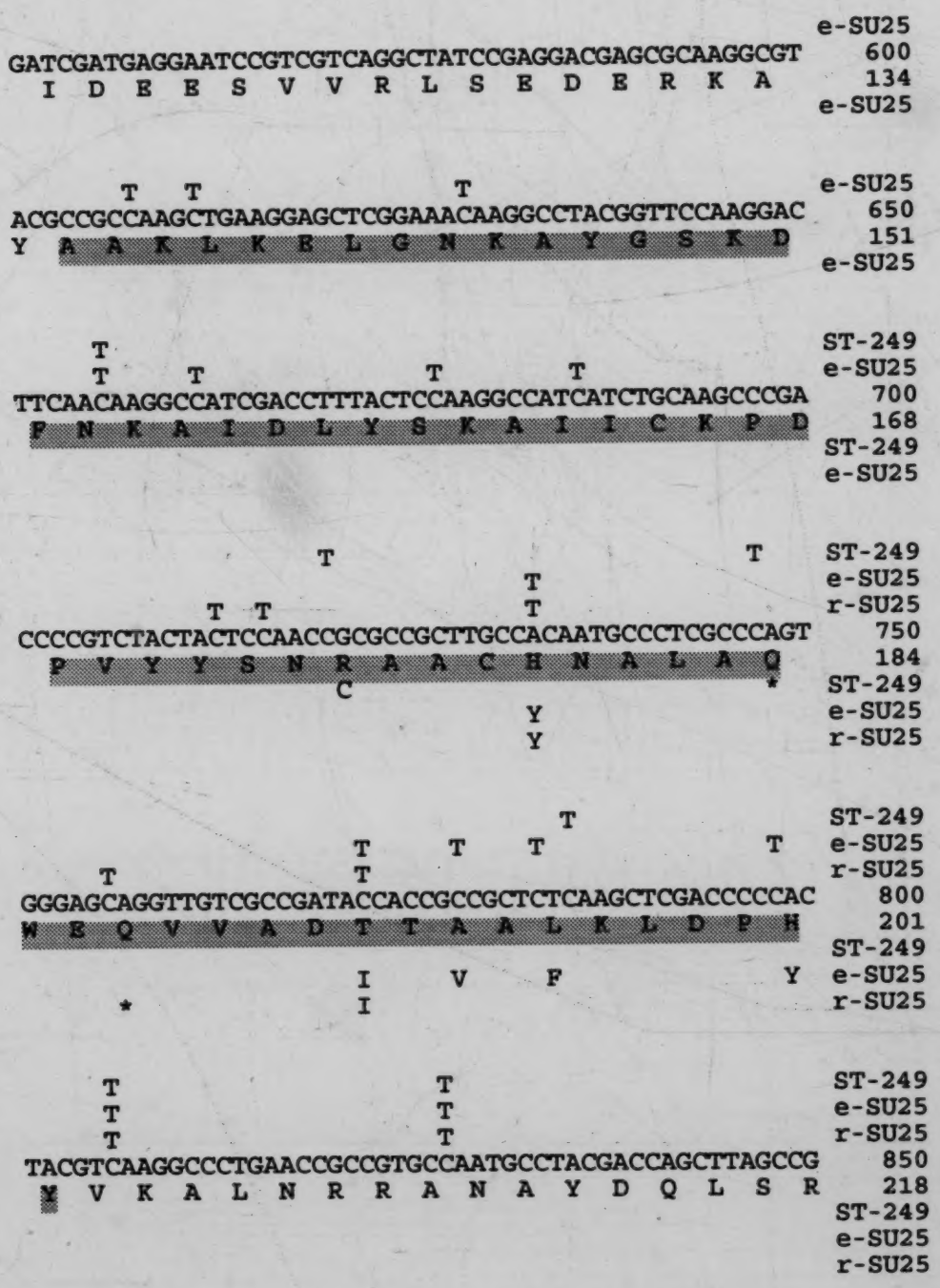
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151
e-SU25
ST-249
T
e-SU25
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700
F N K A I D L Y S K A I I C K P D
168
ST-249
e-SU25
ST-249
T T
e-SU25
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r-SU25
T T T
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750
P V Y Y S N R A A C H N A L A Q
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C *
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r-SU25
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800
W E Q V V A D T T A A L K L D P H
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ST-249
I V F Y
e-SU25
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T T
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T T
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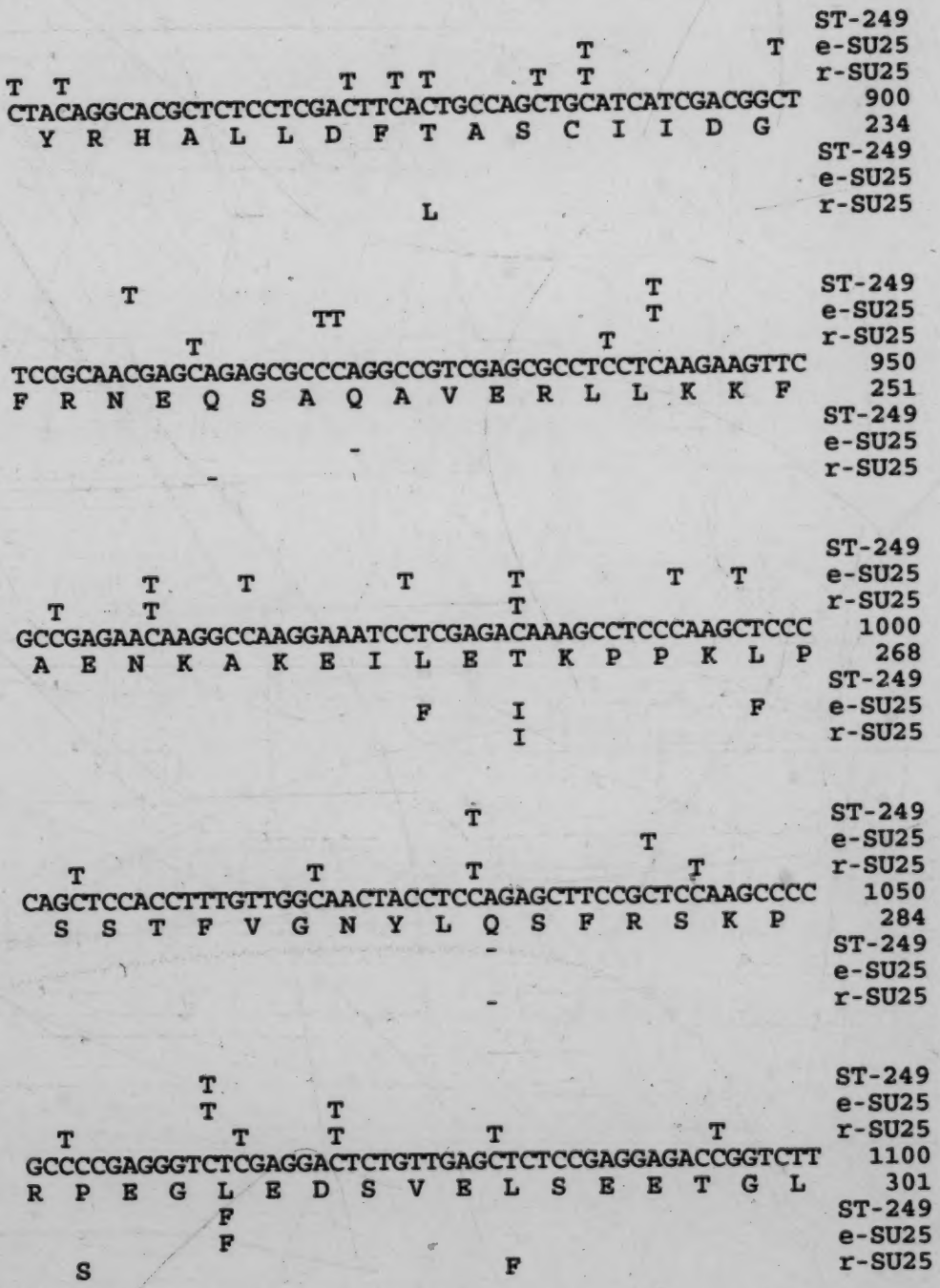
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r-SU25
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F R N E Q S A Q A V E R L L K K F 251
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CAGCTCCACCTTTGTTGGCAACTACCTCCAGAGCTTCCGCTCCAAGCCCC 1050
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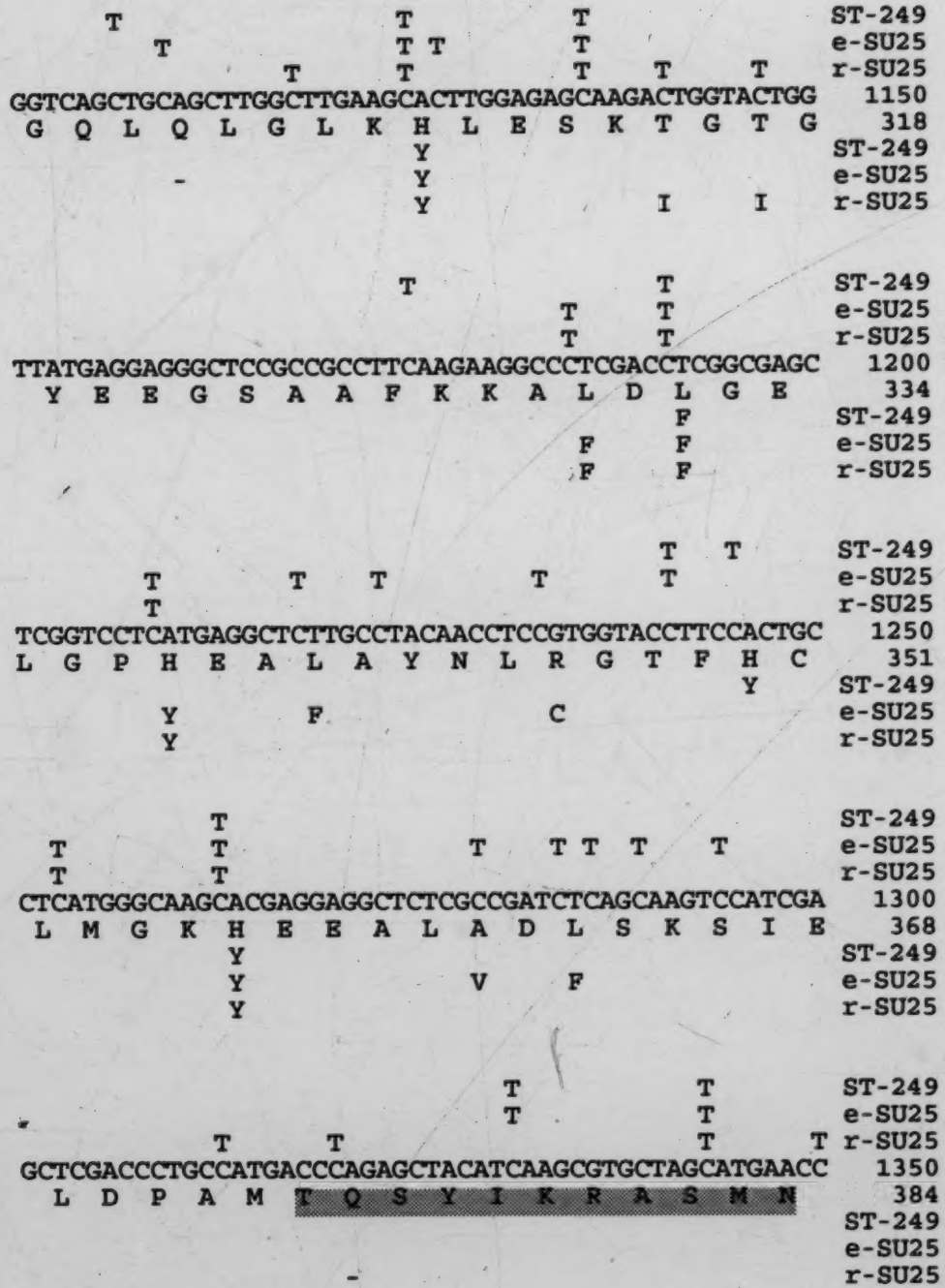
GCCCCGAGGGTCTCGAGGACTCTGTTGAGCTCTCCGAGGAGACCGGTCTT 1100
R P E G L E D S V E L S E E T G L 301


ST-249
e-SU25
r-SU25
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G Q L Q L G L K H L E S K T G T G 318
TTATGAGGAGGGCTCCGCCGCCTTCAAGAAGGCCCTCGACCTCGGCGAGC 1200
Y E E G S A A F K K A L D L G E 334
TCGGTCCTCATGAGGCTCTTGCCTACAACCTCCGTGGTACCTTCCACTGC 1250
L G P H E A L A Y N L R G T F H C 351
CTCATGGGCAAGCACGAGGAGGCTCTCGCCGATCTCAGCAAGTCCATCGA 1300
L M G K H E E A L A D L S K S I E 368
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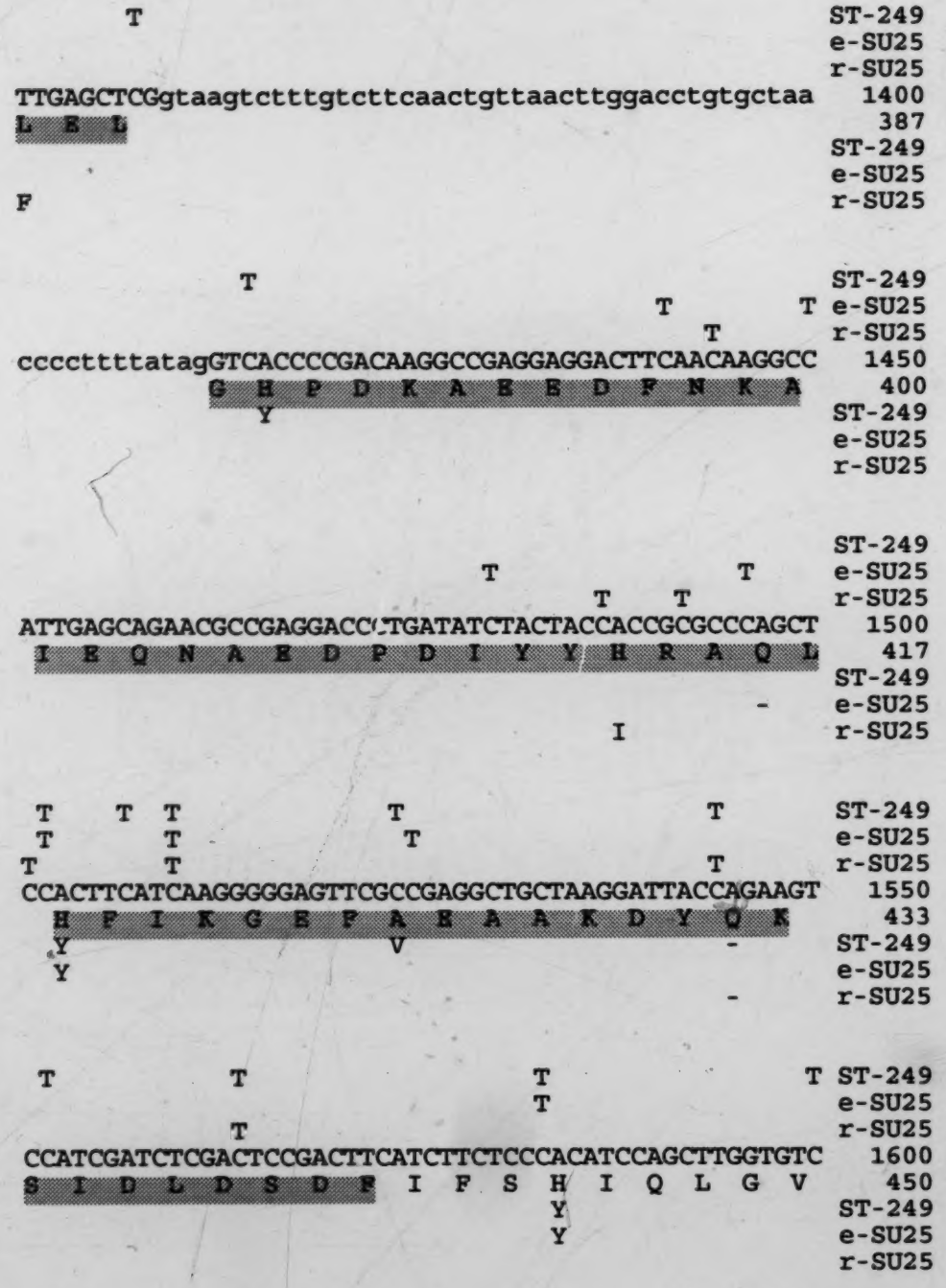
L D P A M T Q S Y I K R A S M N 384


ST-249
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r-SU25
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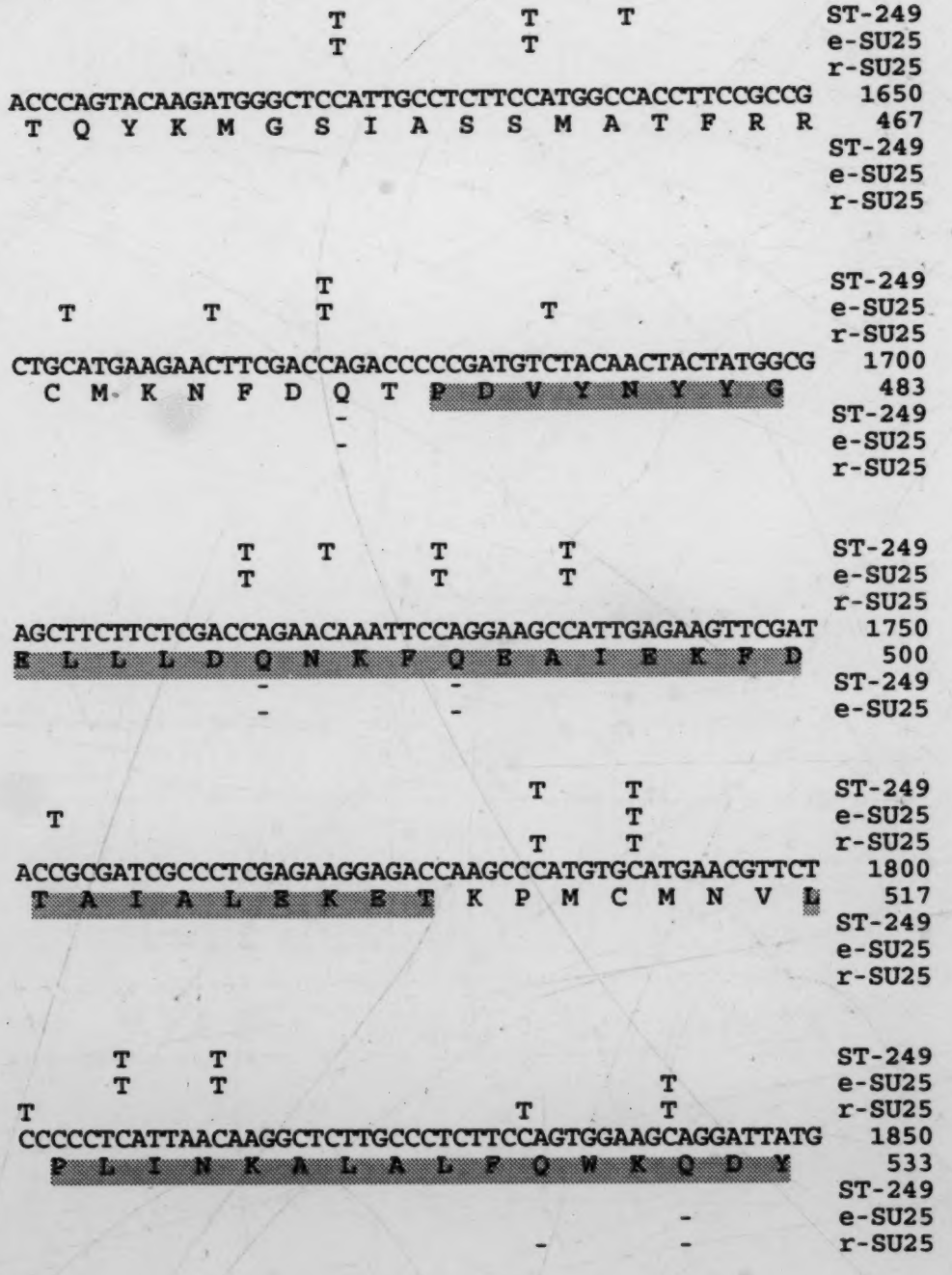
L E L 387
F
cccctttatagGTCACCCCGACAAGGCCGAGGAGGACTTCAACAAGGCC 1450
G H P D K A E E D F N K A 400
Y
ATTGAGCAGAACGCCGAGGACCCTGATATCTACTACCACCGCGCCCAGCT 1500
I E Q N A E D P D I Y Y H R A Q L 417
I
CCACTTCATCAAGGGGGAGTTCGCCGAGGCTGCTAAGGATTACCAGAAGT 1550
H F I K G E F A E A A K D Y Q K 433
Y V -
Y -
CCATCGATCTCGACTCCGACTTCATCTTCTCCCACATCCAGCTTGGTGTC 1600
S I D L D S D F I F S H I Q L G V 450
Y
Y


ST-249
e-SU25
r-SU25
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T Q Y K M G S I A S S M A T F R R 467
CTGCATGAAGAACTTCGACCAGACCCCCGATGTCTACAACTACTATGGCG 1700
C M K N F D Q T P D V Y N Y Y G 483
AGCTTCTTCTCGACCAGAACAAATTCCAGGAAGCCATTGAGAAGTTCGAT 1750
E L L L D Q N K F Q E A I E K F D 500
ACCGCGATCGCCCTCGAGAAGGAGACCAAGCCCATGTGCATGAACGTTCT 1800
T A I A L E K E T K P M C M N V L 517
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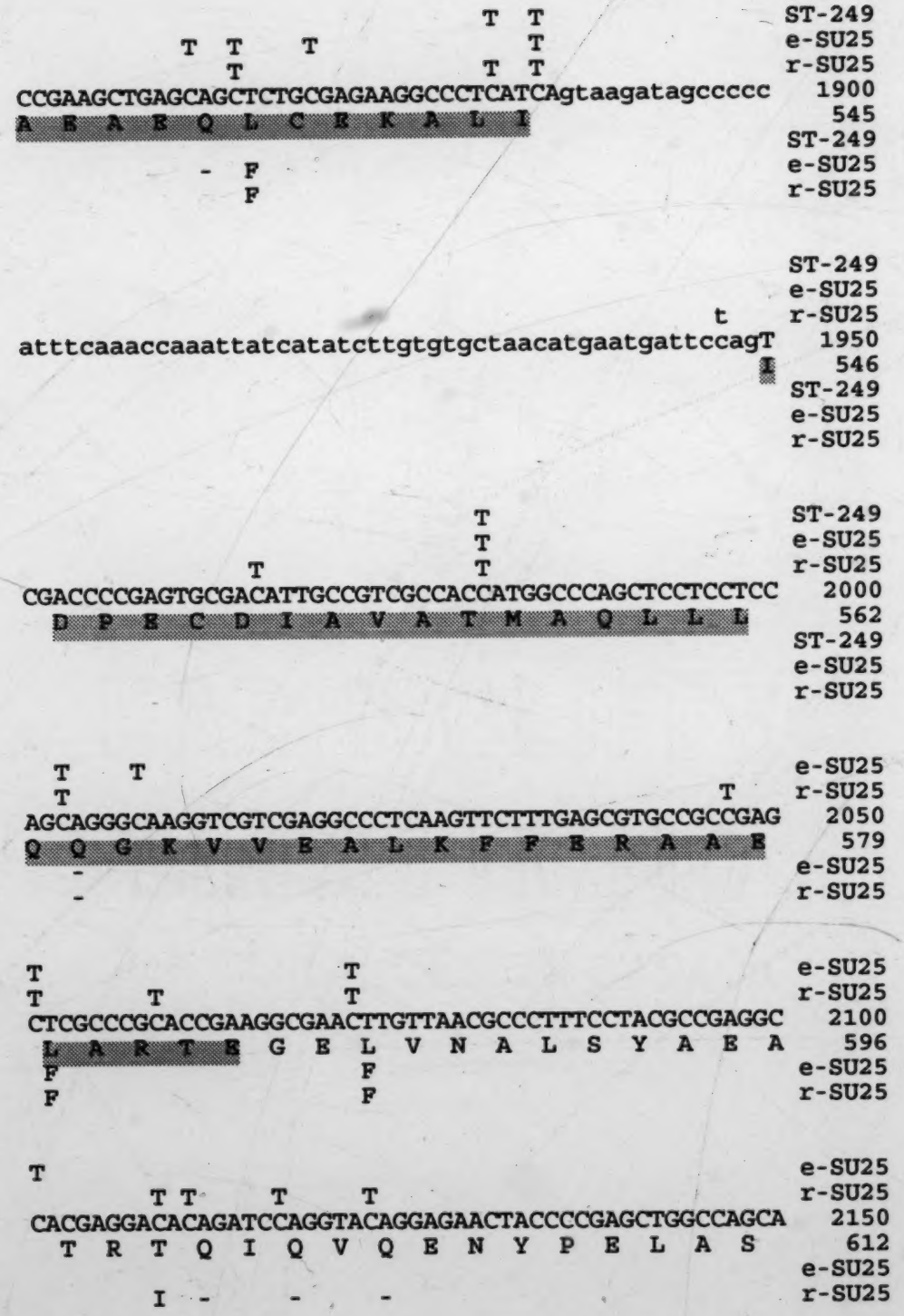
P L I N K A L A L F Q W K Q D Y 533


ST-249
e-SU25
r-SU25
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A E A E Q L C E K A L I 545
atttcaaaccaaattatcatatcttgtgtgctaacatgaatgattccagT 1950
I 546
CGACCCCGAGTGCGACATTGCCGTCGCCACCATGGCCCAGCTCCTCCTCC 2000
D P E C D I A V A T M A Q L L L 562
AGCAGGGCAAGGTCGTCGAGGCCCTCAAGTTCTTTGAGCGTGCCGCCGAG 2050
Q Q G K V V E A L K F F E R A A E 579
CTCGCCCGCACCGAAGGCGAACTTGTTAACGCCCTTTCCTACGCCGAGGC 2100
L A R T E G E L V N A L S Y A E A 596
CACGAGGACACAGATCCAGGTACAGGAGAACTACCCCGAGCTGGCCAGCA 2150
T R T Q I Q V Q E N Y P E L A S 612

```
     T                                                  T    T    r-SU25
AGCTCCAGGGCATGAGCGGTGGTCCCGGCATGCGGTAAAAGCATGTCAAG          2200
K  L  Q  G  M  S  G  G  P  G  M  R                          624
      -                                                      r-SU25


                                                             r-SU25
   T     T  T
ATTACTCTAGCAGCATAAAAAAGGAAGAGAAATAAAGAAAGCGATGAAGT          2250
          ←
TTTGAAACGAAGTAGAAAAGCAGTGTGTTGTACATTAAAAAGGGTTCATT          2300
                                          ⇐
TTTGGTTGTTGCTACTGCTGTAACGGTGTAAGGAGATTGAGGATGTCTGT          2350

TGGCGGTAAGGAGACGCGGGAATGGATGGATGGTGTTTTACATAGGTTGG          2400

CCTGGATGGGATAGGATGTAATGCTTT    2427
```

regions of the gene that are predicted to encode the TPR motifs of the putative TOM70 protein are shown in Fig. 2-7. TPR motifs were identified based on homology to the TPRs found previously in the yeast homologue of TOM70 (Riezman *et al.*, 1983).

The coding sequence of genomic *tom70* was found to contain a discrepancy compared to the published *tom70* cDNA sequence (Steger *et al.*, 1990). The genomic sequence shows an extra 15 bp sequence 5'-GGGAGGCCGAGAAGG-3', located between nucleotides 338 and 339 of the cDNA (exon 2 in the genomic version). The last 12 bp of the extra sequence is a repeat of the previous 12 bp of the *tom70* gene before the extra 15 bp sequence occurs. This extra sequence is in frame and putatively codes for a gly-glu-ala-glu-lys amino acid sequence. To determine if this extra sequence is present in the *tom70* gene of other strains of *N. crassa*, or if it represents an allelic variation of *tom70* unique to strain NCN235, genomic *tom70* DNA was amplified by PCR from DNA isolated from the two other parental strains of the RIP crosses, ST-2-1 and SU-1-1. In addition, four other wild-type strains with non-related genetic backgrounds, NCN7 NCN20, NCN27, and NCN39 were also analyzed. The region containing the extra 15 bp was sequenced from all these PCR products. All strains analyzed contained the extra 15 bp sequence. A possible explanation to account for the absence of this sequence in the cDNA could be an error by the reverse-transcriptase during construction of the cDNA library from which the original cDNA fragment was cloned from. Thus, the 15 bp sequence is likely part of the actual *tom70* coding sequence so that the actual length of the *N. crassa* TOM70 protein is 624 amino acid residues as compared to the previously published length of 619 residues (Steger *et al.*, 1990). In support of this, the cDNA sequence of *P. anserina* contains an amino acid repeat sequence

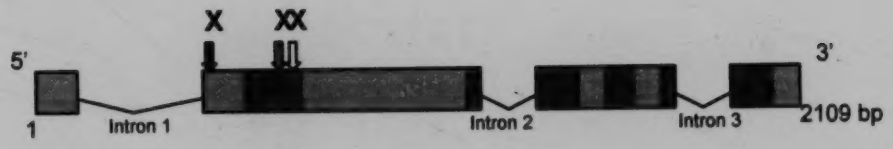


Figure 2-7. Schematic representation of genomic *tom70*. Thick bars denote exons. Thin lines indicate intron regions. Dark gray boxes denote sequences encoding putative tetratricopeptide repeat (TPR) motifs. The first 114 bp of the first exon encode a putative intermembrane space domain. Base pairs 115 to 181 of exon 1 encode the putative membrane-spanning domain. **X**, denotes the first premature stop codon in the RIPed endogenous allele. The black arrow indicates the position of the earliest premature stop codon in the ectopic copy of *tom70*^{RIP} in strain SU25. The light gray arrow indicates the earliest premature stop codon in the endogenous copy of *tom70*^{RIP} in strain ST-249. The white arrow indicates the earliest premature stop codon in the endogenous copy of *tom70*^{RIP} in strain SU25. The first premature stop codons in the endogenous copies of *tom70*^{RIP} in both strains SU25 and ST-249 occur within the second TPR in the N-terminal region. The first premature stop codon present in the ectopic copy of *tom70*^{RIP} in strain SU25 occurs before all seven TPR motifs, and only 5 amino acid residues after the putative membrane-spanning domain.

homologous to the one found in this study (Jamet-Vierny *et al.*, 1997). Curiously, in two of the seven strains of *N. crassa* examined above, a single G to A transition was discovered at the first position of the extra 15 bp sequence. This results in a glutamic acid codon in place of the glycine codon found in the *tom70* gene of the other five strains analyzed. This suggests that natural variation occurs in the coding sequence of *tom70*.

2·3·2·3 DNA sequence analysis of *tom70*^{RIP} alleles

To prove that RIP had occurred in strains SU25 and ST-249 and to characterize the extent of the expected RIP mutations, *tom70* DNA was amplified from the mutant strains by amplification of genomic DNA by PCR using primers LGR15 and LGR16 (Table 2-2). Since it was demonstrated by Southern analysis that ST-249 contained only the endogenous copy of *tom70*, while SU25 also carried the ectopically integrated copy of *tom70* derived from plasmid p72PCR-7, two sets of PCR primers were used with strain SU25. The additional set of primers (LGR13 and LGR14; Table 2-2) was designed to amplify the ectopic copy of *tom70*. Thus, for SU25, two products were generated from these PCR reactions: the resident genomic copy of *tom70* from the *tom70* locus and the ectopic copy of *tom70* derived from p72PCR-7. For strain ST-249, only the endogenous copy of *tom70* was obtained. All the PCR products were sequenced directly. The relative positions of PCR and sequencing primers on the endogenous and ectopic copies of *tom70* are represented schematically in Fig. 2-5.

For the ectopically integrated copy of *tom70* in strain SU25 a total of 82 point mutations were observed in the 1932 base pairs (bp) sequenced (Fig.2-6). Since this ectopic copy of *tom70* was derived from cDNA, it does not contain the extra 15 bp sequence found in the genomic sequence. All of the

mutations were C to T transitions which are characteristic of RIP and clearly demonstrates the strand bias of mutation that is typical of RIP (Cambareri *et al.*, 1989). Of the 82 RIPs identified, 30 resulted in changes in the first position of a codon, 4 resulted in changes in the second position, and 48 resulted in third position changes. The first and second position codon changes resulted in numerous non-conservative amino acid changes, including the generation of a premature stop codon after only 65 amino acid residues (Fig. 2-7). In total, the mutations produced 34 amino acid substitutions.

The 2244 bp sequence from the endogenous genomic copy of *tom70* in SU25 showed a total of 74 RIPs, again all C to T transitions (Fig. 2-6), of which 68 were located within the coding region of the *tom70* gene. Five point mutations were located within 26 bp downstream of the stop codon, and only a single RIP mutation was located within an intron. It was expected that introns would not be affected since they were not present in the cDNA-derived duplication, but RIP is known to extend occasionally into neighboring non-duplicated sequences (Selker, 1990). The RIP mutations in this allele resulted in 25 changes in the first position of a codon, 6 changes in the second position, and 38 changes in the third position. As with the ectopic copy, many of the first and second position codon changes resulted in non-conservative amino acid changes, including a premature stop codon after 181 amino acid residues, and resulted in a total of 31 amino acid substitutions (Fig. 2-7).

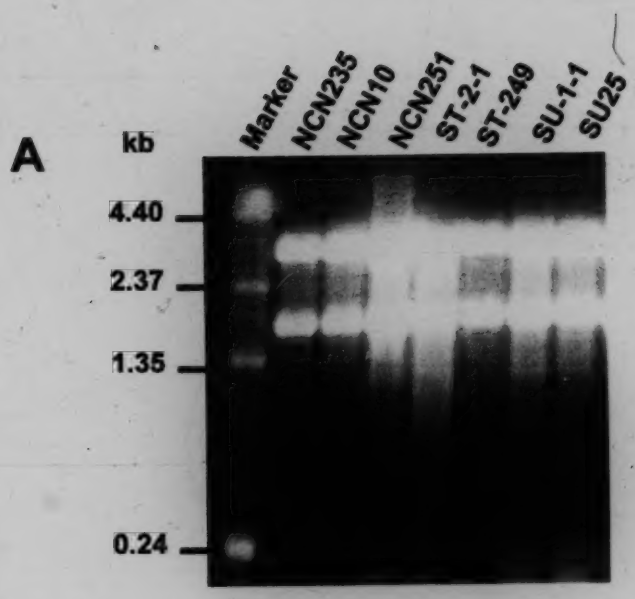
In the 2244 bp sequenced of the endogenous genomic copy of *tom70* in strain ST-249, 46 RIPs were identified all within the coding region of the gene, and all C to T transitions (Fig. 2-6). The RIP mutations in this allele resulted in 16 changes in the first position of a codon, 1 change in the second

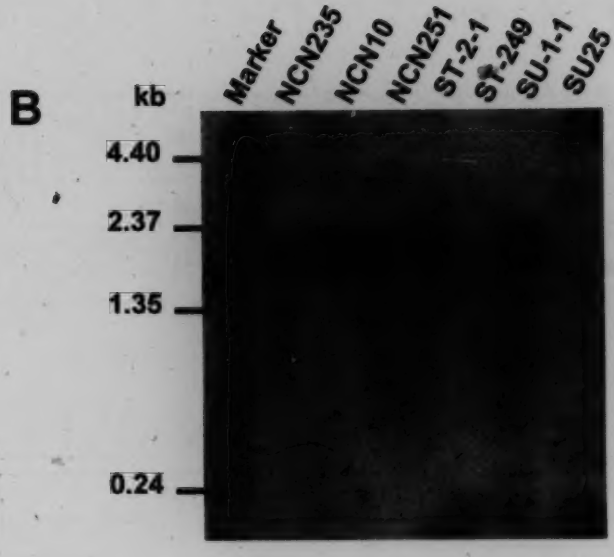
position, and 29 changes in the third position resulting in a total of 16 amino acid substitutions. A premature stop codon was generated after 178 amino acid residues (Fig.2-7). The presence of premature stop codons and several amino acid substitutions prior to the true stop codons suggests the RIPed alleles, if translated, most likely give rise to non-functional truncated gene products. This prediction is supported by the lack of immunologically detectable TOM70 in mitochondria isolated from RIPed strains, on western blots (Section 2·2·1, Fig. 2-3).

2·3·2·4 *tom70*^{RIP} strains do not produce detectable amounts of *tom70* mRNA

Past observations have noted that DNA sequences containing numerous RIP mutations are usually heavily methylated (Singer *et al.*, 1995). Methylation in *N. crassa* has been shown to prevent accumulation of mRNA (Irelan and Selker, 1997). It is thought that methylation in *N. crassa* does not significantly affect transcription initiation, but rather causes RNA polymerase II to stall in the 5' end of genes (Roundtree and Selker, unpublished). To determine if the transcription of *tom70*^{RIP} alleles in strains SU25 and ST-249 is affected by the RIP mutations, a radioactively-labeled *tom70* cDNA sequence derived from p72PCR-7 was used to probe a northern blot of total RNA isolated from both the *tom70*^{RIP} strains and appropriate control strains (Fig. 2-8). The blot shows large amounts of *tom70* mRNA in all control strains, but no detectable *tom70* mRNA in the *tom70*^{RIP} strains. These results indicate that the RIP mutations and/or RIP-induced methylation in the *tom70*^{RIP} alleles of strains SU25 and ST-249 effectively prevent transcription. As a result, the two *tom70*^{RIP} strains are most likely null mutants of *tom70*.

Figure 2-8. *tom70*^{RIP} strains do not contain detectable amounts of *tom70* mRNA. **A**. Total RNA separated by electrophoresis on a 1.8% formaldehyde-agarose gel stained with ethidium bromide. 5 μg of total RNA isolated from indicated strains were loaded per lane. The size of RNA markers is indicated on the left. **B**. Autoradiograph of northern blot of agarose gel shown in panel A. Following electrophoresis, RNAs were blotted onto nylon membrane and probed with a radioactively-labeled 1.9 kb *Hind*III *tom70* cDNA fragment isolated from p72PCR-7 (Fig.2-1).


A
kb
Marker
NCN235
NCN10
NCN251
ST-2-1
ST-249
SU-1-1
SU25
4.40
2.37
1.35
0.24


B
kb
Marker
NCN235
NCN10
NCN251
ST-2-1
ST-249
SU-1-1
SU25
4.40
2.37
1.35
0.24

2·3·2·5 Growth phenotype of *tom70*^{RIP} mutants

Further evidence that the *tom70* gene was inactivated was the distinct growth phenotype of both *tom70*^{RIP} strains SU25 and ST-249. When inoculated in flasks containing solid medium, both strains grew at a slower rate, did not extend hyphae up the sides of the flask, and conidiated very poorly as compared to the wild-type strain NCN235 (Fig. 2-9). Linear hyphal extension of both mutant strains compared to wild-type parental strains was quantitatively analyzed by growth in race tubes at 22°C and 37°C (Fig. 2-10). The rate of mycelial advance in the two *tom70*^{RIP} strains was approximately 65% of the wild-type parental strains at 22°C and 45% of wild-type growth at 37°C. Temperature-sensitive growth defects have also been observed in TOM70 mutants of yeast (Riezman *et al.*, 1983) and *Podospora anserina* (Jamet-Vierny *et al.*, 1997). It would appear that inactivation of the *tom70* gene has a mild, but obvious, effect on the rate of growth of *N. crassa* cells.

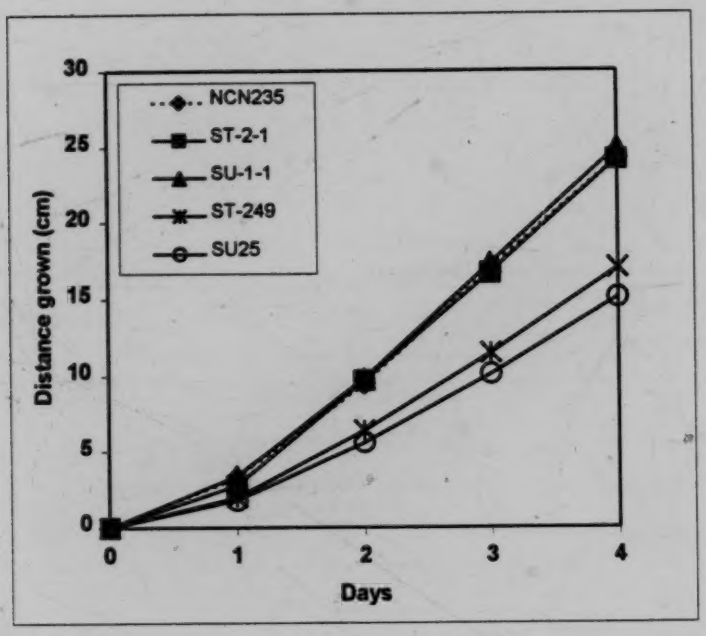
2·3·2·6 Rescue of *tom70*^{RIP} mutant strains

The effects of RIP are not always specific and it is possible that the observed phenotypes of the *tom70*^{RIP} mutant strains might have been the result of mutations within a gene in close proximity to *tom70*. To ensure that the defects in conidiation and growth were due specifically to disruptions within the *tom70* gene, mutant strains SU25 and ST-249 were transformed with the bleomycin-resistance plasmid pLGR10 (Fig. 2-2) which contains the entire *tom70* cDNA. As controls, transformation of each strain was also performed with a plasmid carrying bleomycin-resistance (pAB520; Austin *et al.*, 1990), but lacking a copy of *tom70*. Twenty bleomycin-resistant colonies from each transformation were picked and purified through two rounds of single colony isolation on bleomycin-containing medium to ensure that

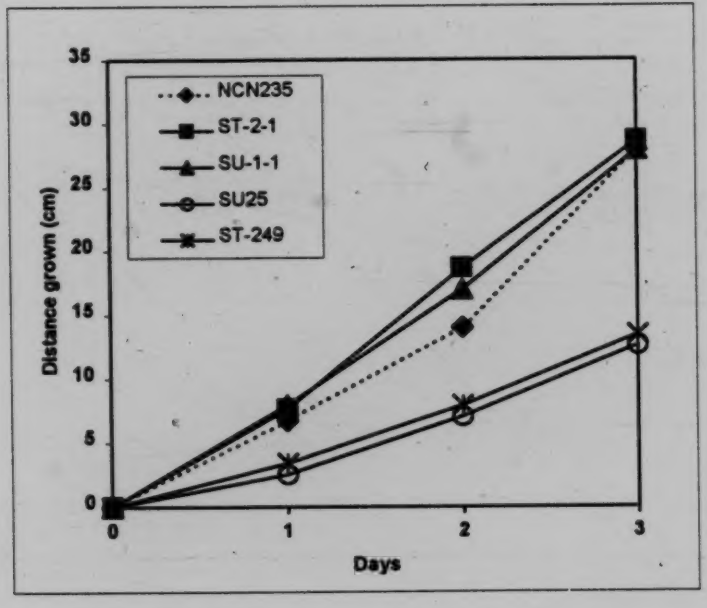
Figure 2-9. *tom70*^{RIP} strains have a defective growth morphology. Conidia from a wild-type and mutant strain were used to inoculate 50 ml of solid medium in each flask. The flask on the left is fully conidiated (NCN235) whereas the strain on the right is poorly conidiated and the mycelia have not efficiently climbed the walls of the flask (SU25). Both strains were incubated at 30°C for 48 h and then at room temperature for 5 days.

Figure 2-10. Hyphal extension rates of wild-type and mutant strains. Strains were inoculated in solid medium race tubes containing sucrose, and allowed to grow over several days. **A**. Growth at 22°C. **B**. Growth at 37°C. Strains NCN235, ST-2-1, and SU-1-1 represent the parental wild-type strains used in the RIP crosses. Strains SU25 and ST-249 lack immunologically detectable TOM70 (Fig.2-3).

A


NCN235
ST-2-1
SU-1-1
ST-249
SU25
Distance grown (cm)
Days
0
5
10
15
20
25
30
0
1
2
3
4

B


NCN235
ST-2-1
SU-1-1
SU25
ST-249
Distance grown (cm)
Days
0
5
10
15
20
25
30
35
0
1
2
3

transformants were homokaryotic. Transformants were allowed to conidiate in solid-medium flasks to determine if the wild-type growth phenotype was restored. Several transformants from the pLGR10 transformation, such as SU25-10-6-1, SU25-10-11-1, and ST-249-10-2-1 displayed significant qualitative increases in hyphal extension and conidiation comparable to wild-type strain NCN235. Some strains were judged to have partially-restored phenotypes. For example, ST-249-10-1-1 had normal levels of hyphal extension, but poor conidiation, while strain ST-249-10-4-1 had poor hyphal extension, but a normal level of conidiation. None of the picked transformants obtained with the bleomycin-resistance plasmid lacking a copy of *tom70* displayed any evidence of restored conidiation or growth rate. Mitochondria from strains displaying a full range of growth phenotypes were isolated and polyclonal antibodies against TOM70 were used to probe western blots of mitochondrial proteins (Fig.2-11). It is evident from the western analysis that only those transformants with immunologically detectable TOM70 had a completely or partially restored growth phenotype. Since plasmid pLGR10 containing the full-length *tom70* cDNA could have integrated anywhere within the genome, it is possible that the site of integration might affect the resulting growth phenotype by disrupting another locus. Alternatively, the ectopically integrated copy of *tom70* could be under the influence of a promoter that results in lower than normal expression. From these data it is evident that the phenotypic characteristics of strains SU25 and ST-249 are due to the loss of the *tom70* gene function.

2·4 Discussion

The phenomenon of RIP was utilized to generate mutants of *tom70* in *N. crassa*. Two strains isolated following RIP crosses, SU25 and ST-249,

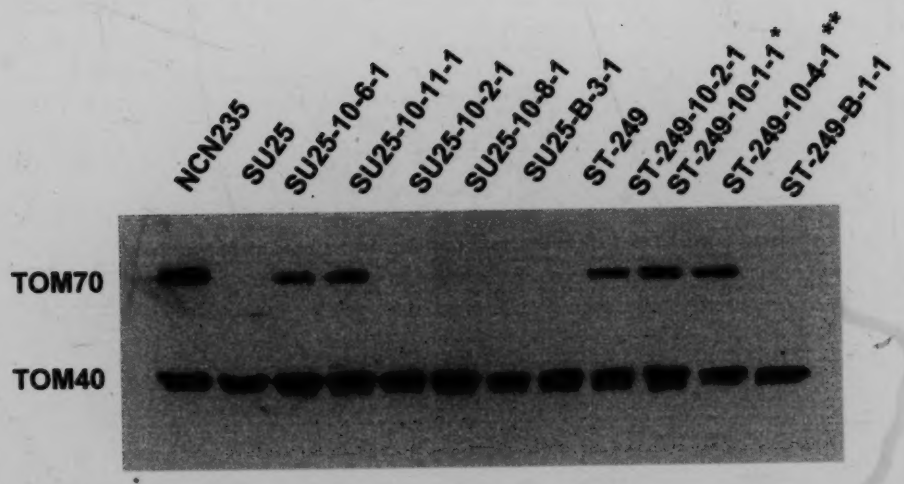


Figure 2-11. TOM70 in rescued transformants of *tom70*^{RIP} strains. Strains SU25 and ST-249 were transformed with either pLGR10 (Fig. 2-2) which contains a bleomycin-resistance gene and a *tom70* cDNA sequence, or with a similar plasmid that does not carry the *tom70* sequence. Only those strains transformed with pLGR10 show the presence of immunologically detectable TOM70 on Western blots. "10" in the strain name denotes it is an isolate of the pLGR10 transformation. "B" in the strain name denotes it is an isolate of the transformation with the bleomycin-resistant plasmid not carrying a *tom70* sequence. 15 μg of protein from isolated mitochondria were loaded per lane. Following electrophoresis, the proteins were electroblotted to nitrocellulose membrane and probed with polyclonal antibodies against TOM70 and TOM40 (control). * Indicates partially rescued phenotype in which a normal level of hyphal extension was restored, but conidiation was poor. ** Indicates partially rescued phenotype in which a normal level of conidiation was restored, but hyphal extension was poor.

were identified as lacking immunologically detectable TOM70 protein on western blots of isolated mitochondria. It was determined that strain SU25 carried both the ectopically integrated copy of *tom70* derived from cDNA and the resident genomic copy of *tom70*, while strain ST-249 carried only the resident genomic copy of the gene. Confirmation that RIP took place within the *tom70* alleles present in strains SU25 and ST-249 was achieved by sequence analysis of PCR products of the target gene amplified from genomic DNAs. Numerous C to T transitions, which are characteristic of RIP, were identified within all three *tom70* alleles analyzed. The premature stop codons generated after 65, 181, and 178 in the ectopic copy of SU25, endogenous copy of SU25, and endogenous copy of ST-249, respectively, may result in non-functional truncated protein products that may be rapidly degraded. All three stop codons occur within the first third of the protein which would effectively eliminate the major portion of the putative cytosolic domain of TOM70 (Fig. 2-7). It is this cytosolic domain that is thought to interact with preproteins directly.

The possibility that truncated protein products are inserted into the mitochondrial outer membrane and retain a low level of activity is effectively eliminated by the results of the northern blot analysis of *tom70*^{RIP} strains SU25 and ST-249 (Fig. 2-8) which show no appreciable amounts of *tom70* mRNA produced in these strains. It is apparent that the endogenous and ectopic copies of *tom70* in strain SU25 which contain 75 and 82 RIPs, respectively, and the endogenous copy of ST-249, which carries 46 RIPs, are sufficiently altered to induce heavy methylation of *tom70* sequences and eliminate transcription of *tom70*.

The possibility that the phenotypic effects observed in strains SU25 and ST-249 are due to alterations in other genes adjacent to the RIPed *tom70*

gene is effectively eliminated by the observation that immunologically detectable TOM70 and normal growth rate and levels of conidiation can be restored upon transformation of mutant strains with plasmids containing *tom70* cDNA.

The existence of viable *N. crassa* strains that lack TOM70 demonstrates that the protein is not essential in this organism as was initially suspected based on the viability of *tom70* deletion strains in yeast. The relatively mild growth defects observed in both *tom70*^{RIP} strains (Figs. 2-9, 2-10) are comparable to the minor growth defects observed in *tom70* deletion strains of yeast (see Section 1·2·3·3). In *Podospora anserina*, another species of filamentous fungi, a frameshift mutation in TOM70 affecting the last 97 amino acids of the protein results in an altered germination phenotype and a decrease in the mycelium aerial hyphae (Jamet-Vierny *et al.*, 1997). In addition, this mutant displays a thermosensitive growth phenotype similar to that found in *tom70*-deletion yeast strains. The similarities in growth phenotypes of *tom70* mutant strains in three different organisms suggest that the protein performs similar functions in each. The simplest explanation for the observed phenotypes would be an alteration in import efficiency of specific preproteins that depend upon TOM70 as a mitochondrial import receptor. Presumably, those preproteins that utilize TOM70 for import into mitochondria would bind the mitochondrial surface less efficiently in the absence of TOM70, but can ultimately enter the mitochondrion at a slower rate that normal using the TOM20-TOM22 receptor subcomplex (more on this in Chapter 3). This may result in depletion of certain components needed for proper mitochondrial function. This defect may be exacerbated at higher temperatures, which increases the stress on the organism. However, other effects cannot be ruled out entirely since *tom70* mutant strains also have

altered mitochondrial morphology (see Section 3·3·1). The next chapter will describe a biochemical investigation of TOM70-deficient cells focusing on the functional role of TOM70 in the mitochondrial preprotein import process.

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Chapter 3: Inactivation of *tom70* in *N. crassa* causes specific defects in mitochondrial protein import and morphology

3·1 Introduction

As discussed in Section 1·1, normal mitochondrial function depends upon the ability of nuclear-encoded preproteins to be imported across mitochondrial membranes and sorted to the correct sub-mitochondrial compartment. The translocase of the outer mitochondrial membrane, or TOM complex, is made up of receptor components that have large domains exposed to the cytosol and more membrane embedded protein components that are thought to make up a putative translocation channel or general insertion pore. TOM70 is a component of the complex that plays a relatively specialized role (Söllner *et al.*, 1990). Biochemical studies of TOM70 involving immunoinhibition or protease digestion of TOM70 have demonstrated that only specific preproteins, such as AAC and related carrier proteins, and cytochrome c_1, recognize and bind to TOM70 in isolated mitochondria (Schlossmann *et al.*, 1994; Söllner *et al.*, 1990; Steger *et al.*, 1990). Import of these TOM70-dependent proteins is significantly decreased, but not abolished when TOM70 is cleaved or bound with antibody. TOM70-dependent preproteins are thought to be transferred to the TOM20-TOM22 sub-receptor complex prior to translocation through the pore (Lill *et al.*, 1996).

To better understand the role of TOM70 in the mitochondrial import process, it is important to examine genetic mutants *in vivo* in which the *tom70* gene has been effectively inactivated. *In vitro* import studies of TOM70-deficient yeast mitochondria showed no drastic impairment in import of preproteins possessing a cleavable presequence, but significant defects in the import of AAC and related carrier proteins (Schlossmann *et al.*, 1996). A

frameshift mutation in the TOM70 homologue of *Podspora anserina* results in a growth defect similar to that found in the yeast deletion mutant, and enlarged mitochondria when the mutant strain is grown at high temperatures (Jamet-Vierny *et al.*, 1997). Import of mitochondrial preproteins was not investigated in the *P. anserina* mutant.

In this chapter, the TOM70-deficient strains of *N. crassa* described in Chapter 2 were analyzed with respect to phenotypic consequences of the absence of functional TOM70 on mitochondrial morphology and mitochondrial protein composition, and preprotein import into mitochondria. The results establish a specialized role for TOM70 in preprotein import into mitochondria. TOM70 may also play a role in the maintenance of mitochondrial size and structure.

3·2 Materials and Methods

3·2·1 Strains and growth conditions

Strains of *N. crassa* used in this study were: (i) NCN235; (ii) SU25; (iii) ST-249 (for genetic details see Chapter 2, Table 2-1). Liquid cultures were grown at 25°C with vigorous aeration in Vogel's minimal medium containing appropriate supplements (Davis and De Serres, 1970). Strain NCN235 was grown for 16 h whereas strains SU25 and ST-249 were grown for 20 h.

3·2·2 Biochemical procedures

The following procedures were used: Immunoblotting according to Good and Crosby, (1989), detection by chemiluminescence utilizing the LumiGLO substrate (Kirkegaard and Perry Laboratories, Gaithersburg, MD, product information); quantification by laser densitometer using a GS-670 Imaging Densitometer; *in vitro* transcription of mRNA from vectors carrying

cDNA of mitochondrial precursor proteins utilizing the Promega Ribomax system (Madison, WI, product information); *in vitro* translation of precursor proteins using [^{35}S]-methionine as radioactive label utilizing the Promega Rabbit Reticulocyte Lysate system (product information); alternatively the Promega T'N'T Reticulocyte Lysate system was used to generate precursor proteins (product information); protein determination using bovine serum albumin (BSA) as a standard was performed with the Coomassie dye binding assay (Bio-Rad, Hercules, CA, product information); spectral analysis of cytochromes was according to Bertrand and Pittenger, (1972).

3·2·3 Isolating mitochondria from TOM70-deficient strains of N. crassa

Mitochondria were originally isolated according to the standard procedure of Pfanner and Neupert, (1985), employing 250 mM sucrose in the mitochondrial isolation buffer. However, modifications were introduced because the mitochondrial outer membranes of strains SU25 and ST-249 were found to be unusually fragile. Since mutant mitochondria appeared enlarged, or "bloated" (see Section 3·3·1), it was reasoned that mutant mitochondria would shrink, or become more stable, under increased osmotic pressure and that this might help preserve outer membrane integrity during the isolation procedure. Thus, the isolation of mitochondria was tried with SEMP (sucrose; 1 mM ethylenediaminetetraacetic acid (EDTA); 10 mM 3-(N-morpholino) propanesulfonic acid (MOPS), KOH, pH 7.2; 2 mM phenylmethylsulfonyl fluoride (PMSF)) isolation buffers containing sucrose between 500 mM and 1M. In addition, the duration of manual grinding with quartz sand was reduced to 15-20 s. Mitochondrial isolation using a SEMP isolation buffer made to a sucrose concentration of 1 M was found to substantially increase the likelihood of obtaining intact mitochondrial

preparations, but did not completely prevent the outer membrane of mutant mitochondria from breaking. All experiments that required intact mitochondria were isolated with buffers containing 1 M sucrose. Sucrose concentrations higher than 1 M were not utilized as it was not known what effect the increased osmotic pressures would have on the stability of wild-type mitochondria, or what effect the increased viscosity of the buffer would have on the efficiency of *in vitro* import of radioactively-labeled precursors into isolated mitochondria.

3·2·4 Mitochondrial outer membrane integrity assay

Mitochondria were isolated as described in Section 3·2·4 with the following changes: filter harvested mycelia was ground in the presence of SEMP buffer made to 500 mM sucrose containing 2 mM PMSF. Isolated mitochondria (30 μg protein) were suspended in 100 μl of SEM buffer and incubated with graded concentrations of proteinase K ranging from 2.5 to 40 μg/ml for 15 min at 0°C. Samples were then treated with an equal volume of 25% TCA, incubated at 0°C for 5 min, and spun for 2 min at room temperature in an Eppendorf 5415 microcentrifuge to remove supernatant. The pellets were shaken in the presence of 1 ml cold acetone for 5 min and re-spun to remove supernatant. Pellets were air-dried and solublized in 2X cracking buffer (1.25 M Tris-HCl, pH 6.8, 10% (w/v) SDS, 10% (v/v) β-mercaptoethanol, 20% (v/v) glycerol, 2% (w/v) bromophenol blue). Protein was analysed by SDS-PAGE and fluorography.

3·2·5 Protein import into isolated mitochondria

Mitochondria were isolated as in Section 3·2·3. A typical import reaction consisted of 5μl freshly isolated mitochondria (30 μg protein) in SEM

buffer, 1-2 µl (depending upon the quality and intensity of the radioactivity) rabbit reticulocyte lysate containing radioactively labeled precursor proteins, 143 µl import buffer (1 M sucrose, 3% (w/v) fatty acid free BSA, 80 mM KCl, 5 mM $MgCl_2$, and 10 mM MOPS/KOH, pH 7.2) to a total volume of 150 µl. Samples were supplemented with an energy mix (2 mM ATP, 5 mM NADH, 10 mM creatine phosphate, and 100 µg of creatine kinase). Import was performed at 15 or 25°C at 2, 8, and 20 min intervals. After chilling on ice, designated samples were treated with 100 µg/ml of proteinase K for 15 min at 0°C. Protease digestion was halted by the addition of 2 mM PMSF from a freshly prepared 200 mM stock in ethanol followed by a dilution with 1 ml SEMP buffer. Mitochondria were re-islolated and solublized in 2X cracking buffer. To demonstrate that the observed import was surface-receptor mediated, designated samples were treated with 20 µg/ ml trypsin for 25 min at 0°C prior to import to remove surface proteins form the mitochondrial outer membrane. Trypsin digestion was halted with 20-fold excess (w/v) of soy-bean trypsin inhibitor. Mitochondria were re-isolated by centrifugation for 5 min at 14,000 rpm at 4°C in an Eppendorf 5414 microcentrifuge. Radioactive imported proteins were subjected to SDS-PAGE, autoradiography, and quantified by phosphor-imager scanner. The level of import that occurred in trypsin-digested mitochondria ("bypass") was subtracted from the amount of precursor imported for quantification.

Protein insertion assays for TOM22 precursor consisted of freshly isolated mitochondria 5µl (30 µg protein), 3µl rabbit reculocyte lysate containing radioactively labeled precursor protein, and 142 µl import buffer in a total volume of 150 µl. Samples were supplemented with an energy mix (2 mM ATP and 5 mM NADH). Import was performed at 25°C for 10 min. After chilling on ice, designated samples were immediately treated with 15 µg/ml

proteinase K for 15 min at 0°C. Protease digestion was halted as above. Designated samples were also pre-treated with trypsin as above. Mitochondria were solublized in 2X cracking buffer and imported radioactive protein was analysed by SDS-PAGE and autoradiography. Quantification was performed by a phosphor-imager.

3·2·6 Electron microscopy

1 ml of cell suspension from 12 h liquid cultures was fixed in 1.5% $KMnO_4$ for 30 min at room temperature followed by repeated washings with distilled water (cycles of gentle agitation of cells in water to allow excess stain to diffuse out of cells; centrifugation; pouring off water; adding fresh water) until the liquid was colourless. Cells were centrifuged for 2 min at 14,000 at room temperature in an Eppendorf 5414 microcentrifuge and supernatant was removed. Cells were re-suspended in 0.05 M sodium cacodylate buffer containing 2% glutaraldehyde and 30% sucrose, incubated at 0°C for 30 min, and then post-fixed in 1% (w/v) OsO_4 and 1.5% (w/v) $K_2Cr_2O_7$. All samples were post-stained in 1% (w/v) uranyl acetate for 16 h at room temperature and sent to R. Bhatnagar (University of Alberta, Edmonton, Alberta) for analysis. Samples were prepared for electron microscopy by dehydration in a graded ethanol series and embedded in an epoxy resin. Ultra-thin sections were cut with a diamond knife and examined in a transmission electron microscope.

3·3 Results

3·3·1 TOM70-deficient cells have an abnormal mitochondrial morphology

The mitochondrial morphology in TOM70-deficient cells was examined in the mutant strain SU25 and compared to mitochondria in wild-type strain NCN235 (Fig. 3-1). Several differences were observed between mitochondria from the two strains. First, mutant mitochondria were considerably larger than their wild-type counterparts. In addition, mitochondria from the TOM70-deficient strain were elongated and had distorted membrane envelopes with numerous kinks compared to the relatively smooth and more spherical shape of wild-type mitochondria. Second, cristae were disorganized and occasionally truncated in mutant mitochondria whereas normal mitochondria carried linear, ordered cristae. Third, the number of mitochondrial profiles per cell was noticeably less in mutant mitochondria than in wild-type cells, but the distribution of organelles did not seem to be altered (S. Seiler, personal communication). The mitochondrial morphology of TOM70-deficient cells differed from that of TOM20- (Harkness *et al.*, 1994) and TOM22-depleted cells (Nargang *et al.*, 1995). In the latter two cases, mitochondria decreased in size, increased in the number of profiles per cell, and were virtually devoid of inner membrane cristae. Enlarged mitochondria have also been observed in a *tom70* mutant strain of *Podospora anserina* (Jamet-Vierny *et al.*, 1997).

Western blot analyses performed early in the study of isolated mitochondria from the *tom70*^{RIP} strain SU25 showed variable decreased amounts of the soluble intermembrane space protein, cytochrome *c* heme lyase (CCHL). Conceivably, the absence of TOM70 could cause the mitochondrial outer membrane to be more fragile and more susceptible to damage than wild-type mitochondria during the opening of cells to isolate mitochondria. A broken mitochondrial outer membrane would permit the

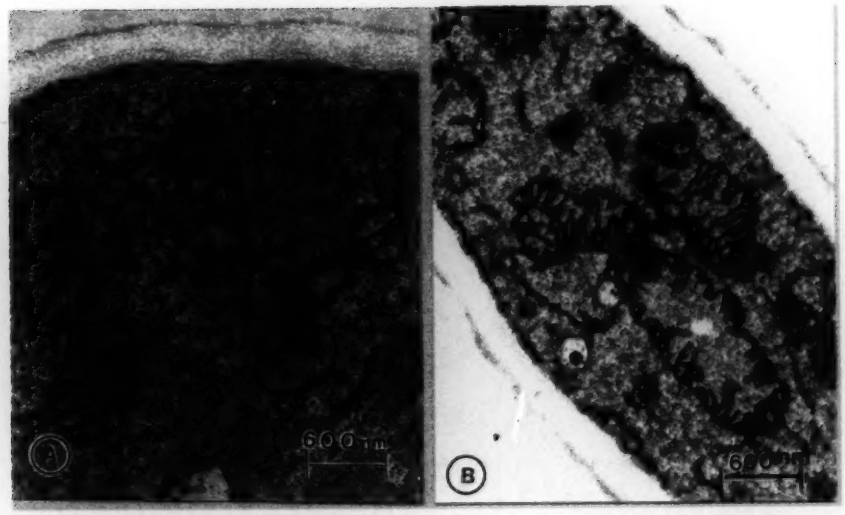


Figure 3-1. TOM70-deficient mitochondria are enlarged and have an abnormal ultra-structure. Electronmicrographs of cells grown for 12 h and fixed with $KmnO_4$. **A.** Wild-type strain NCN235. **B.** TOM70-deficient strain SU25.

soluble CCHL to leak out of the mitochondrion or allow proteases into the intermembrane space. To test the fragility of TOM70-deficient mitochondria, the organelles were isolated from strains SU25 and NCN235 using a SEMP isolation buffer with a sucrose concentration of 500 mM. Isolated mitochondria were then suspended in SEM buffer containing the same concentration of sucrose and exposed to graded concentrations of proteinase K ranging from 2.5 to 40 μg/ml. These protease-treated mitochondria were then examined for the presence of specific mitochondrial protein markers using antibodies against the protease-sensitive outer membrane protein TOM22, the soluble intermembrane space protein CCHL, and the mitochondrial matrix chaperone Hsp70. When mitochondria are undamaged, proteins that reside in the matrix and intermembrane space are resistant to extra-mitochondrial proteases. Decreases in the amount of CCHL or Hsp70 in the presence of extra-mitochondrial proteases would indicate that the integrity of the outer membrane and inner membrane, respectively, was compromised. The results of the western blot analysis clearly demonstrated that while the relative amounts of proteins within normally protease-resistant compartments of wild-type mitochondria remained constant at all concentrations of proteinase K tested, TOM70-deficient mitochondria showed decreasing levels of CCHL with increasing concentrations of proteinase K (Fig. 3-2). The amount of Hsp70 in the matrix of mutant mitochondria was not affected by increasing protease concentrations. These data indicate that the outer membrane of TOM70-deficient mitochondria is more fragile than the outer membrane of wild-type mitochondria. The lack of TOM70 therefore not only contributes to abnormally enlarged mitochondria with disorganized inner membrane cristae, but also alters the mitochondrial outer membrane

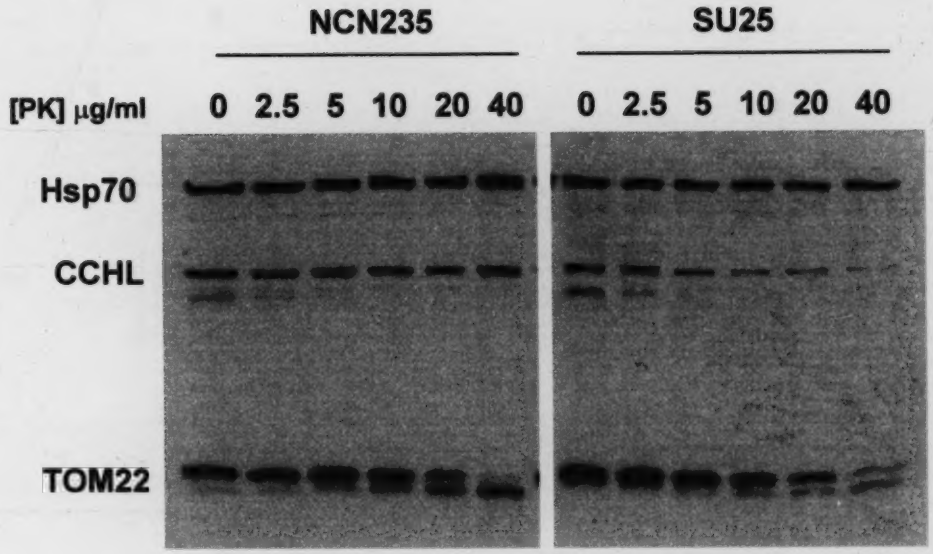


Figure. 3-2. TOM70-deficient mitochondria have weakened mitochondrial outer membranes that are more fragile than wild-type mitochondria during the standard isolation procedure. Mitochondria from strains NCN235 and SU25 were isolated in SEMP buffer containing 500 mM sucrose and resuspended in SEM buffer of the same sucrose concentration. Aliquots of mitochondria containing 30 μg of protein were then incubated with the indicated concentrations of proteinase K (PK) for 15 min at 0ºC. Protease treatments were stopped by the addition of 1 μl of 0.2 M PMSF. Samples were immediately treated with 12.5% TCA to precipitate protein. TCA-precipitated pellets were suspended in 2X cracking buffer and loaded onto a polyacrylamide gel. After electrophoresis, protein was electroblotted to nitrocellulose membrane and probed with polyclonal antibodies raised against mtHsp70, the N-terminus of CCHL, and the C-terminus of TOM22.

composition in such a way that decreases its ability to withstand the normal procedure of mitochondrial isolation.

3·3·2 TOM70-deficient mitochondria have a slightly altered protein composition

To examine TOM70-deficient mitochondria for changes in steady-state levels of individual mitochondrial proteins, mitochondria were isolated from NCN235, SU25, and ST-249 cells and analysed by immunostaining with antibodies to a number of mitochondrial proteins (Fig. 3-3, Table 3-1). It was of interest to determine if the amounts of other components of the TOM complex were altered when TOM70 was deficient. The steady state level of the primary component of the translocation pore TOM40 was not affected in mitochondria lacking TOM70. In contrast, the steady-state amounts of TOM22 and TOM20 were increased by about 30% in mutant mitochondria. Conceivably, the levels of TOM20 and TOM22 could be adjusted in a coordinated fashion in response to the absence of TOM70 since the two components are thought to act in concert (Harkness *et al.*, 1994). The increased steady-state levels of TOM20 and TOM22 may result from increased transcription and/or translation, or more efficient import and assembly into the mitochondrial outer membrane in response to the absence of TOM70. The steady-state level of TIM17, a component of the TIM complex, displayed no changes in mutant mitochondria. Individual non-TOM complex proteins representing components found in the four different sub-mitochondrial compartments were also analysed (Fig. 3-3, Table 3-1). The levels of porin, CCHL, and Hsp70 in TOM70-deficient mitochondria were similar to levels found in wild-type. Surprisingly, even steady-state levels of AAC, whose import has been shown to be dramatically influenced by TOM70

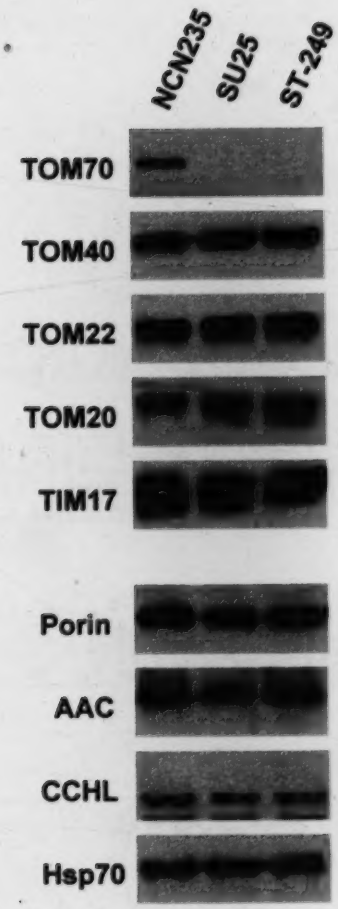


Figure 3-3. Protein composition of TOM70-deficient mitochondria. Mitochondria were isolated from strains NCN235, SU25, and ST-249. 7.5 to 50 μg of mitochondrial protein were analysed by SDS-PAGE and immunoblotting for the relative amounts of the indicated proteins. AAC, ADP/ATP carrier; CCHL, cytochrome *c* heme lysae, Hsp70, heat-shock protein of 70 kDa.

Table 3-1. Abundance of mitochondrial proteins in TOM70-deficient mitochondria given as a percentage of the levels in wild-type (NCN235) mitochondria.

	N.crassa strain		
Protein	**NCN235**	**SU25**	**ST-249**
TOM20	100	136±13	129±11
TOM22	100	136±4	134±4
TOM40	100	101±4	101±9
TOM70	100	N.D.	N.D.
TIM17	100	107±12	106±14
AAC	100	99±14	92±14
CCHL	100	93±2	89±2
Porin	100	99±13	102±11
Hsp70	100	97±3	97±8

Mitochondria were isolated from the indicated strains and mitochondrial protein (7 to 35 μg) was analysed for the relative amounts of various individual proteins by SDS-PAGE and immunoblotting using the LumiGLO chemiluminescence detection system (Kirkegaard and Perry Laboratories). Quantification was performed by scanning densitometry. Data are given relative to the values obtained for wild-type strain NCN235 (100%). Values are the mean average of 4 to 6 different experiments (± sample standard deviation). N.D., not detectable.

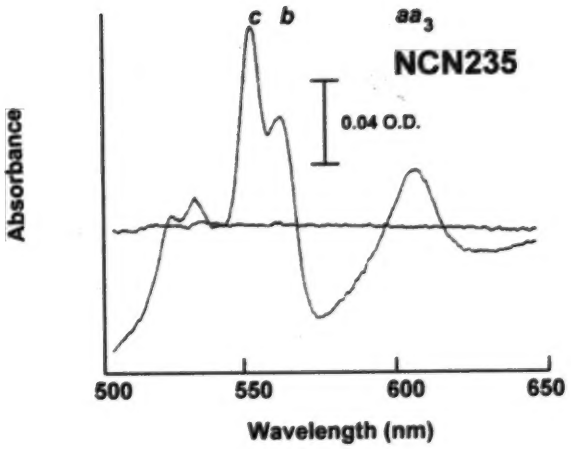
(Steger *et al.*, 1990; Hines *et al.*, 1990), appeared normal in mutant mitochondria.

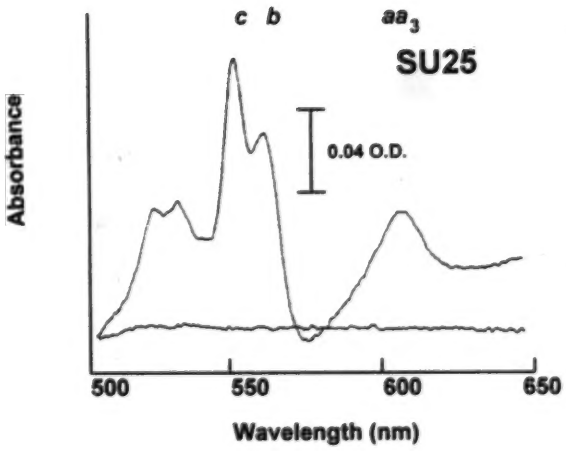
Absorption difference spectra of mitochondria (Bertrand and Pittenger, 1972) isolated from strains NCN235 and SU25 showed that the amounts of cytochrome *b*, and *c* appeared virtually unchanged, while the amount of cytochrome aa_3 was slightly diminished in mutant mitochondria (Fig. 3-4). The height of the absorption peak for cytochrome aa_3 in mutant mitochondria was down by approximately 25% compared to the corresponding peak in the wild-type spectrum. Since some components of cytochromes aa_3 and *b* are encoded by mtDNA, the absence of TOM70 may cause a minor defect in the expression of certain mitochondrially-encoded proteins, or a specific affect on the import of one or more nuclear-encoded components. The slightly altered level of cytochrome aa_3 may contribute to the slower growth rate of TOM70-deficient strains.

3·3·3 Import of specific preproteins is affected by the absence of TOM70

To assess the consequences of inactivation of TOM70 on the translocation of individual mitochondrial preproteins, import into isolated mitochondria was measured. Mitochondria isolated from strains NCN235 and SU25 were incubated with radioactively-labeled, *in vitro* synthesized preproteins. Import was assessed for a number of preproteins representing components from all four sub-mitochondrial compartments. These included preproteins of the β-subunit of the F_1-ATPase ($F_1\beta$; Rassow *et al.*, 1990) and the α-subunit of the matrix processing peptidase (α-MPP; Schneider *et al.*, 1990) from the matrix space; the ADP/ATP carrier (AAC; Söllner *et al.*, 1990) from the inner membrane; cytochrome *c* heme lyase (CCHL; Lill *et al.*, 1992) from the intermembrane space; and porin (Kleene *et al.*, 1987) and TOM22

Figure 3-4. TOM70-deficient mitochondria have a slightly altered cytochrome content. Mitochondria were isolated from strains NCN235 and SU25 and, examined by differential absorption spectrophotometry (Bertrand and Pittenger, 1972). The absorption maxima of cytochromes aa_3, *b*, and *c*, at wavelengths of 608, 560, and 500 nm, respectively, are indicated. A baseline reading is shown for each spectrum. Equivalent amounts of mitochondrial protein (5 mg/ml) were used to obtain each spectrum.


c b
aa3
NCN235
0.04 O.D.
Absorbance
500
550
600
650
Wavelength (nm)


c b
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SU25
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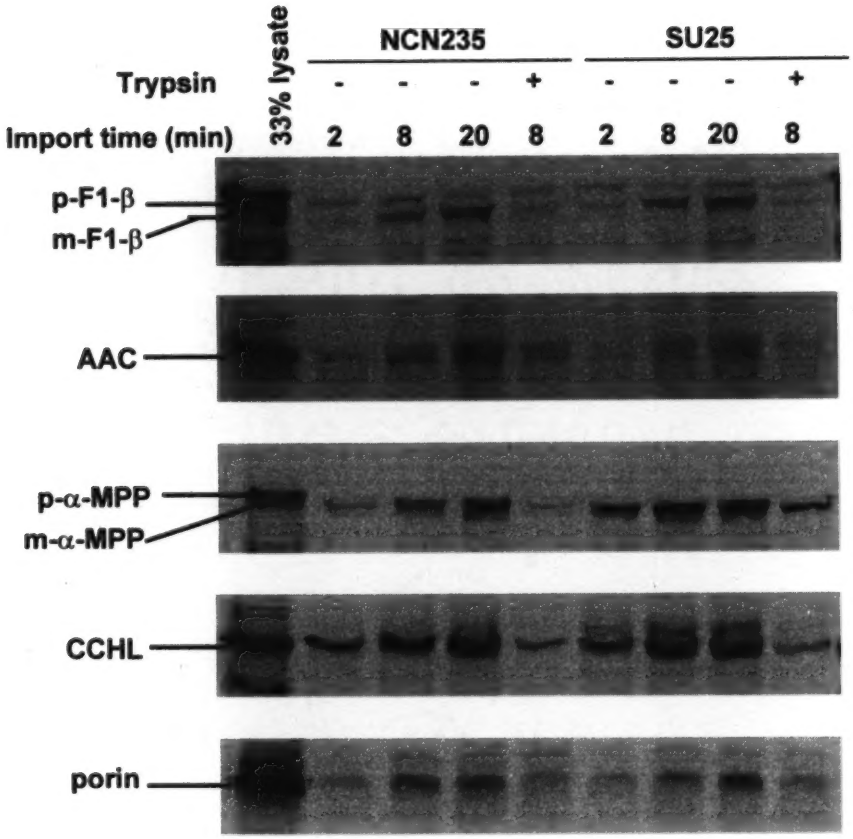
(Keil and Pfanner, 1993) from the outer membrane. The $F_1\beta$ and α-MPP precursors contain cleavable presequences while precursors for AAC, CCHL, porin, TOM20, and TOM22 do not. Import of TOM complex-dependent preproteins into TOM70-deficient mitochondria was at or near wild-type levels for most of the preproteins tested with two exceptions (Fig. 3-5, panels A, B, Fig. 3-6). Import of AAC into isolated mitochondria was reduced by almost 60%. In contrast, the import of α-MPP was increased approximately 2-fold in the absence of TOM70.

The difficulties in isolating mitochondria with intact outer membranes from TOM70-deficient strains (See sections 3·2·3 and 3·3·1) led to a concern that import assays might not be a reliable evaluation of preprotein import into TOM70-deficient mitochondria as it is known that many precursors can be imported directly via the TIM complexes (Hwang and Schatz, 1989; Segui-Real *et al.*, 1993). To insure that isolated mitochondria were intact during import, the intermembrane space protein CCHL was co-imported with other precursors, and the level of CCHL import was used to judge if the outer membrane of isolated mitochondria was broken. Alternatively, steady-state levels of CCHL in isolated mitochondria were examined by immunostaining after import was completed (not shown). CCHL is known to utilize the TOM20-TOM22 receptor sub-complex for import into mitochondria (Lill *et al.*, 1992) and therefore its import efficiency was predicted not to decrease in the absence of TOM70.

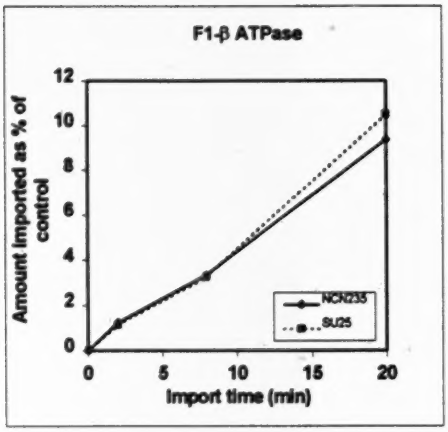
The import data described above are in good agreement with previous findings that have shown significant decreases in AAC import in the absence of functionally active TOM70. AAC import into TOM70-deficient yeast mitochondria has been shown to be diminished 60% (Schlossmann *et al.*, 1996) to 70% (Steger *et al.*, 1990) (Fig. 3-5 B). Even more severe affects on

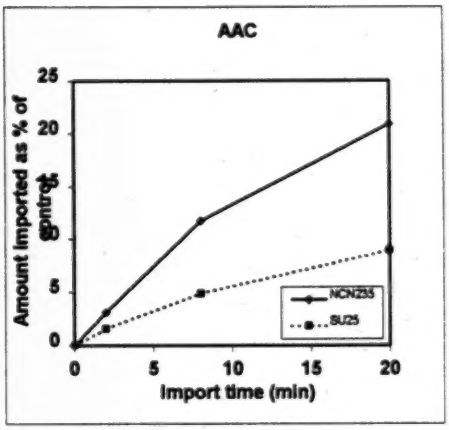
Figure 3-5. Import of mitochondrial preproteins into TOM70-deficient mitochondria. **A**. Import of preproteins into protease-resistant compartments. Radioactively labeled preproteins were incubated in import buffer (see Section 3·2·5 for details) with freshly isolated mitochondria (30 μg protein) from strains NCN235 and SU25. Import was conducted at 15°C for precursors of cytochrome *c* heme lyase (CCHL), porin, and the α-subunit of the matrix processing peptidase (α-MPP), and at 25°C for precursors of the ADP/ATP carrier (AAC), and the β-subunit of the F_1-ATPase (F_1β-ATPase). The import temperature was increased for the latter two precursors because the high sucrose concentration (1 M) of the import buffer impeded their import into mitochondria. The other precursors were not affected in this way. After the indicated times import was terminated by transfer to 0°C and immediate addition of proteinase K (100 μg/ml). Mitochondria were re-isolated by centrifugation, and samples were analysed for imported protein by SDS-PAGE and autoradiography. The control lane (33% lysate) contains 33% of reticulocyte lysate containing radioactively-labeled preproteins used in each import reaction. Selected samples were treated with trypsin (20 μg/ml) for 25 min at 0°C prior to import to remove surface receptors, and incubated with radioactively labeled precursor for 8 min at the appropriate temperature. Samples were post-treated with proteinase K as for the non-trypsin treated samples described above. These samples represent the level of 'bypass' import. **B**. Time course plots of preprotein import into isolated mitochondria from strains NCN235 and SU25. Plotted values represent the amount of radioactively-labeled precursor imported via surface receptors (by-pass import was subtracted) as a percentage of the total amount of precursor present in each import reaction. Quantification of import blots was performed by phosphor-imaging. Plotted values are the average of 4 different experiments.

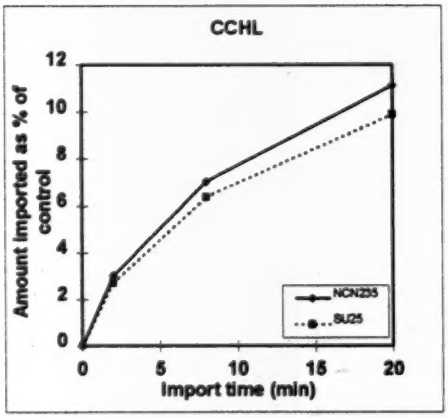
A

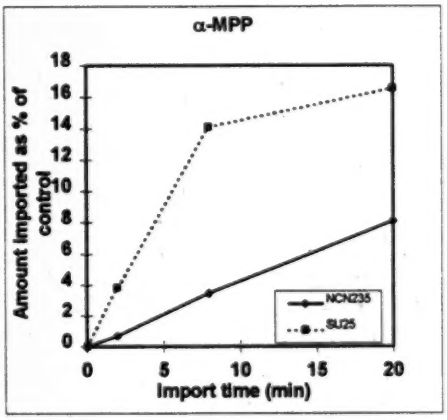

33% lysate
NCN235
SU25
Trypsin
- - - + - - - +
Import time (min)
2 8 20 8 2 8 20 8
p-F1-β
m-F1-β
AAC
p-α-MPP
m-α-MPP
CCHL
porin

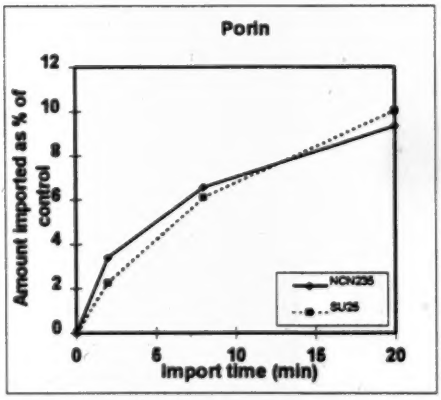
B


F1-β ATPase
Amount imported as % of control
Import time (min)
NCN235
SU25


AAC
Amount imported as % of control
Import time (min)
NCN235
SU25


CCHL
Amount imported as % of control
Import time (min)
NCN235
SU25


α-MPP
Amount imported as % of control
Import time (min)
NCN235
SU25


Porin
Amount imported as % of control
Import time (min)
NCN235
SU25

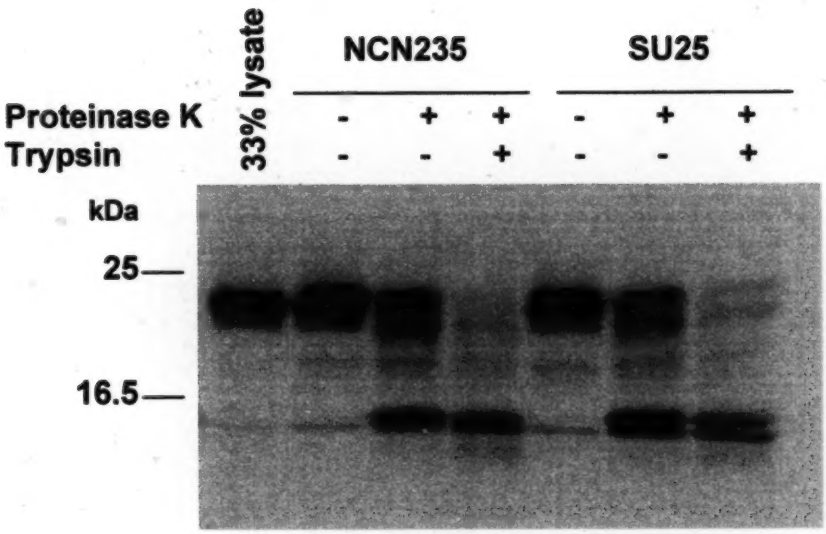


Figure 3-6. Import of TOM22 into TOM70-deficient mitochondria is comparable to that in wild-type mitochondria. Radioactively labeled TOM22 precursor was incubated in import buffer (see Section 3·2·5) with freshly isolated mitochondria (30μg) from strains NCN235 and SU25. Import was conducted at 25°C for 10 min and was terminated by transfer to 0°C and immediate treatment with 15 μg/ml proteinase K. Further analysis of samples was as in Figure 3-5. Import was estimated from the fragmentation pattern of TOM22 produced by the post-import proteinase K treatment. Correct insertion of TOM22 into the mitochondrial outer membrane gives a characteristic pattern of TOM22 degradation (Keil and Pfanner, 1993). Predigestion of surface receptors with trypsin results in no import of TOM22 and complete degradation of unimported TOM22 precursor by proteinase K. Control lane (33% lysate) represents 33% of the total amount of radioactive precursor present in each import reaction.

AAC import were demonstrated with *N. crassa* mitochondria blocked by antibodies directed at yeast TOM70, but steric hindrance may account for the increased deficiency of AAC import into these mitochondria. The finding that import of certain preproteins with cleavable presequences was increased in the absence of TOM70 had been previously seen in yeast (Schlossmann *et al.*, 1996). The rate of import of $F_1\beta$ was enhanced by 30% in *tom70*-disruption mitochondria and up to 60% in *tom70/tom71*-double disruption mitochondria. It is conceivable that the slightly increased levels of TOM20 and TOM22 (Fig. 3-3; Table 3-1) or some unknown factor resulting from a deficiency in TOM70 accounts for the increase in α-MPP import in *N. crassa* and $F_1\beta$ import in yeast. In summary, these data demonstrate the requirement of TOM70 for efficient import of AAC into mitochondria. However, in agreement with previous data (Harkness *et al.*, 1994; Kiebler *et al.*, 1993; Söllner *et al.*, 1990) the TOM20-TOM22 subcomplex is also able to import AAC, though at a lower efficiency than in mitochondria containing functional TOM70.

The finding that TOM22 insertion and assembly is relatively unaffected by the lack of functionally active TOM70 (Fig. 3-6) does not agree with previous findings. *N. crassa* mitochondria incubated with antibodies directed against either TOM70 or TOM20 had demonstrated a significant decrease of properly inserted and assembled TOM22 in the outer membrane showing dependence for TOM22 insertion on both TOM22 and TOM70 (Keil and Pfanner, 1993). The discrepancy between these data and the present study may be attributable to steric hindrance caused by the use of antibodies to block interactions between preproteins and the import receptor.

During the import studies described above, it was noted that the amount of "bypass" import into mutant mitochondria was altered for some of

the precursors tested (Table 3-2). Bypass import is defined as protein import that occurs in the absence of protease-sensitive outer membrane surface components (Pfaller *et al.*, 1989). Bypass import occurs at a low efficiency, and preproteins are thought to enter the mitochondria at a later stage of the import reaction, possibly by making direct contact with components of the general insertion pore. However, at least a portion of bypass import might also be due to a small residual level of intact receptors, or fragments of the receptors that remains following treatment with trypsin. Bypass import for α-MPP was increased almost four-fold in TOM-70 deficient mitochondria compared to wild-type. Bypass levels for CCHL, $F_1\beta$, and porin were comparable in both wild-type and mutant mitochondria, while AAC bypass in TOM70-deficient mitochondria was decreased by 80% compared to wild-type. Upon trypsin digestion, TOM70 is normally cleaved to release a 60 kDa fragment leaving a 12kDa fragment in the outer membrane. This smaller fragment represents the N-terminal portion of the protein, including the membrane anchor and a short sequence of ~30 amino acid residues protected from extra-mitochondrial proteases (Schlossmann *et al.*, 1994). It is conceivable that the 12 kDa protease-resistant fragment still embedded in the outer membrane of wild-type mitochondria after trypsin digestion may affect bypass import rates by affecting the organization of the other sub-units of the TOM complex. However, a complete lack of the TOM70 protein created genetically might result in a translocase complex with slightly altered properties that are revealed under bypass import conditions. An effect on the structural organization of TOM40 due to alterations in TOM22 has been previously observed (Rapaport *et al.*, 1998). The 12 kDa fragment may also play a direct role in the bypass import of those preproteins dependent upon

Table 3-2. Percentage of 'bypass' import for various precursors in wild-type (NCN235) and TOM70-deficient mitochondria (SU25).

	N. crassa strain	
Precursor	NCN235	SU25
$F_1\beta$	17±6	19±3
αMPP	10±1	33±11
AAC	29±6	16±4
CCHL	18±7	18±5
Porin	28±1	29±5

Isolated mitochondria from strains NCN235 and SU25 (30μg of protein) were treated with trypsin (20μg/ml) for 25 min at 0°C to remove surface receptors. Trypsin-treated mitochondria were incubated with radioactively-labeled precursor for 8 min under import conditions described in Section 3·2·5. Samples were analysed by SDS-PAGE and autoradiography for the amount of precursor imported into a protease-resistant compartment. Quantification was performed by a phosphor-imager. Data is given as a percent of import compared to a corresponding sample that had been subjected to only a mock trypsin pre-treatment prior to incubation with precursor for 8 min. Values are the mean average of 4 different experiments (± sample standard deviation).

TOM70 as suggested by the decrease in AAC bypass import in TOM70-deficient mitochondria.

3·4 Discussion

The phenomenon of RIP was used to inactivate the *tom70* gene of *N. crassa* and strains containing mitochondria that lack TOM70 were isolated. Deficiency of TOM70 results in defects in the import of specific precursors, mitochondrial structure, growth rate, and conidiation. Mitochondrial protein content in TOM70-deficient cells does not differ dramatically from wild-type. This is true even for AAC, a protein whose import was shown to be reduced by about 60% by the loss of TOM70 in this study (Section 3·3·3). It may be that for mitochondria to function, a relatively standard level of AAC, and/or other related carrier proteins that depend on TOM70 for import (Schlossmann *et al.*, 1994; Schlossmann *et al.*, 1996), must be present. Thus, the growth rate of the TOM70-deficient cells is determined by the rate at which the mitochondria can accumulate AAC in sufficient levels. The increased steady-state levels of both TOM20 and TOM22 in mutant mitochondria may serve as a compensatory response to the loss of TOM70 resulting in an AAC import efficiency that is still lower than wild-type levels, but helps in reaching a normal steady-state level of the protein in mitochondria. In contrast, yeast mitochondria that lack TOM70 have abnormally low steady-state levels of AAC and normal levels of TOM20 compared to wild-type mitochondria (Schlossmann *et al.*, 1996) suggesting that there may be inherent differences between the function and mode of action between yeast and *N. crassa* homologues of TOM70. The increases in TOM20 and TOM22 levels in TOM70-deficient mitochondria may also help to explain the significant

increase in α-MPP import into these mitochondria since α-MPP precursor has a cleavable presequence and is dependent upon the TOM20-TOM22 import pathway. However, this would also suggest that import of the $F_1\beta$-ATPase should be increased in a similar fashion. Interestingly, import of $F_1\beta$-ATPase into TOM70-deficient yeast mitochondria is increased above that of wild-type levels (Schlossmann *et al.*, 1996). Why this precursor behaves differently in TOM70-deficient mutants of the two organisms, and why other precursors that utilize TOM20-TOM22 for translocation across the mitochondrial outer membrane did not also show significant increases in import efficiency into TOM70-deficient *N. crassa* mitochondria can not be readily explained.

It is difficult to determine if the altered mitochondrial morphology of TOM70-deficient mitochondria is directly due to the lack of TOM70, or due to a secondary effect on an unknown component whose import, and/or assembly, depends upon TOM70. The possibility that TOM70 plays a direct role in mitochondrial structure cannot be dismissed. It was originally suggested that TOM70 might mediate interactions between mitochondria and components of the cytoskeleton (Steger *et al.*, 1990), since the protein contains seven tetratricopeptide repeat motifs. Proteins harbouring these motifs mediate protein-protein interactions, including interactions with cytoskeletal components (Goebl and Yanagida, 1991; Radanyi *et al.*, 1994), but at present it is not known what function these motifs perform in mitochondria. In yeast, cells harbouring certain temperature-sensitive actin alleles exhibited defects in mitochondrial shape and movement during meiotic division (Smith *et al.*, 1995) demonstrating interaction between cytoskeletal components and mitochondria. For *N. crassa*, it has been documented that mitochondrial distribution is mediated by microtubules (Steinberg and Schliwa, 1993).

A number of different mitochondrial outer membrane proteins required for normal mitochondrial morphology have been identified in yeast (Mdm10p, Mmm1p, and Mdm12p; Berger and Yaffe, 1996). Loss of any of these proteins results in normal tubular-shaped mitochondria being converted to one or a few giant spheres. Cells lacking Mdm10p or Mdm12p grow extremely slowly on non-fermentable carbon sources. Inactivation of the yeast *yme1* gene, which encodes a mitochondrial inner membrane protein, also results in temperature-sensitive growth on non-fermentable carbon sources, the absence of mitochondrial DNA, and enlarged and misshapen mitochondria (Campbell *et al.*, 1994). The growth phenotype observed in all cases resembles that of TOM70 mutants in both *N. crassa* and yeast. The abnormal mitochondrial morphology is similar to that observed in TOM70 mutant cells of *N. crassa*. Similar observations have also been made in the filamentous fungus, *Podospora anserina*, in which TOM70 mutant cells displayed enlarged misshappen mitochondria and temperature-sensitive growth defects (Jamet-Vierny *et al.*, 1997). Taken together, the data suggest that TOM70 could affect mitochondrial size and structure. The effect could be achieved directly via interaction with cytoskeletal components at the mitochondrial surface. Alternatively, the import of one or more proteins that directly interact with the cytoskeleton, or contribute in some way to determining ultrastructure could be decreased in TOM70-deficient mitochondria. It is also apparent that the abnormal morphology of mutant mitochondria does not result in general defects in mitochondrial preprotein import since, of all the precursors tested in this study, only the import of the AAC precursor was affected.

Mitochondria that lack TOM70 were also characterized by a weaker mitochondrial outer membrane compared to wild-type. This phenotype has

also been reported in TOM70-deficient strains of yeast (D. Court, personal communication). Again, the presence of TOM70 could be directly responsible for contributing to outer membrane integrity. Alternatively, TOM70 could act as a specific preprotein receptor for other mitochondrial precursors that are responsible for maintaining membrane composition and integrity. It is also possible that the increased bypass import of certain preproteins into mutant mitochondria may be the result of the abnormal state of the outer membrane.

The significant reduction in the ability to import AAC precursor is a prominent characteristic of mitochondria lacking functional TOM70. The import of all other precursors tested, irrespective of the presence or absence of a cleavable presequence, was not reduced by the absence of TOM70. This emphasizes that the function of TOM70 in preprotein import is highly specific for AAC and, by inference, related carrier proteins, and is consistent with previous observations (Hines *et al.*, 1990; Söllner *et al.*, 1990; Harkness *et al.*, 1994; Schlossmann *et al.*, 1994; Schlossmann *et al.*, 1996). Why do the carrier proteins require their own specialized preprotein receptor? One possibility may be that since AAC and related carriers are the most abundant mitochondrial proteins (Klingenberg, 1984), they may demand a specific import mechanism into mitochondria. It should be noted that only a small fraction of the total mitochondrial proteins that utilize the TOM complex for import into the mitochondria were analyzed in this and other studies. Therefore it is possible that other proteins that have yet to be tested or identified utilize TOM70 for import across the mitochondrial outer membrane.

Previous experiments suggested that TOM70 provided the docking site for preproteins that were targeted by mitochondria import stimulating factor (MSF) in an ATP-dependent manner (Hachiya *et al.*, 1993). Import studies in this thesis and in investigations in yeast (Schlossmann *et al.*, 1996) suggest

that not all precursors that require cytosolic ATP for import have to enter mitochondria via TOM70. For instance, the import of the β-subunit of the F_1-ATPase and the α-subunit of the matrix-processing peptidase was not affected in TOM70-deficient mitochondria, although these preproteins require ATP for import (Wachter *et al.*, 1994). Similarly, the import of porin strongly depends on ATP (Kleene *et al.*, 1987), but import of this precursor into mutant mitochondria was not affected. Whether MSF, or an MSF-like chaperone is required for targeting preproteins to TOM70 in *N. crassa* remains to be seen as no such protein has been identified to date.

3·5 References

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Chapter 4: General Discussion

4·1 Future investigations

The technique of RIP is a useful tool for inactivating genes in *N. crassa*. With the previous development of 'sheltered RIP' (Harkness *et al.*, 1994), the phenomenon can be used to destroy both essential and non-essential genes. The use of RIP has allowed for the functional analysis of mutant strains deficient in TOM70. These studies have reinforced the specialized role of TOM70 in preprotein import of carrier proteins into mitochondria and demonstrated that the protein is not essential for viability in *N. crassa*. The absence of TOM70 does not disrupt the assembly of other TOM complex constituents or that of any other mitochondrial proteins examined by western analysis.

Perhaps the most interesting question arising from this study is what accounts for the increased size and abnormal ultrastructure of TOM70-deficient mitochondria. The data presented in this thesis and in related investigations of TOM70 in *N. crassa* and other organisms (Jamet-Vierny *et al.*, 1997; Schlossmann *et al.*, 1996; Söllner *et al.*, 1990) suggest that the function of TOM70 may not be limited to that of a preprotein receptor for AAC and other related carrier proteins. It may also have a role in mitochondrial structure and/or it may act as a receptor for another class of protein involved in determining mitochondrial morphology. The possibility of a role for TOM70 in mitochondrial morphology is supported by the presence of the seven TPR motifs present in the protein. Future studies could utilize site-directed mutagenesis to destroy the predicted 'knob' secondary structure formed by the residues of the TPRs in TOM70 (Goebl and Yanagida, 1991). This would effectively destroy the motif's protein-binding capability. From such TOM70

TPR mutants, it might be determined if the motifs represent the actual site for preprotein binding and/or the site of interaction with components of the cytoskeleton. TPR mutations in TOM70 of any organism have yet to be investigated.

The generation of TOM70-deficient mutants, as well as the availability of other receptor-deficient mutants can allow for more in-depth analysis of the initial steps of protein translocation across the mitochondrial outer membrane. The TOM70 mutant strains generated in this study, may be useful for identifying other components, if any, involved in the TOM70-dependent import pathway in *N. crassa*. One strategy to do this would involve the construction of a TOM70 protein with a hexahistidinyl (his_6) tag at the C-terminus. By transforming TOM70-deficient strains with a gene encoding a his_6-tagged TOM70 protein, rescued strains of *N. crassa* that carry only TOM70 protein with a his_6-tag could be obtained. Isolation of TOM70 from these strains using metal-chelate affinity columns might reveal the presence of other proteins associated with TOM70. Such proteins might include the *N. crassa* homologues of TOM71 and TOM37, which have thus far only been identified in yeast (Bömer *et al.*, 1996; Gratzer *et al.*, 1995; Schlossmann *et al.*, 1996). Alternatively, whole cell cytosolic preparations from *N. crassa* cells carrying hexahistidinyl-tagged TOM70 could be passed through an affinity column to identify possible cytosolic factors involved in TOM70-dependent mitochondrial preprotein import or cytoskeletal components that complex with TOM70.

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